THE UNIVERSITY OF ALBERTA

INTERLEUKIN 2 IN THE IN VITRO GENERATION AND IN VIVO EFFECTIVENESS OF TUMOR SPECIFIC T LYMPHOCYTES

by

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(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING 1984

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR GORDON B MILLS

TITLE OF THESIS INTERLEUKIN 2 IN THE IN VITRO GENERATION AND IN VIVO EFFECTIVENESS OF TUMOR SPECIFIC T LYMPHOCYTES

DEGREE FOR WHICH THESIS WAS PRESENTED DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED SPRING 1984

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled INTERLEUKIN 2 IN THE IN VITRO GENERATION AND IN VIVO EFFECTIVENESS OF TUMOR SPECIFIC T LYMPHOCYTES submitted by GORDON B MILLS in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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Date December 9, 1983

ABSTRACT

In order to respond to a foreign challenge, cells of the immune system must recognize the pathogen as foreign and must also receive a "second signal". Both signals are required to induce the proliferation and differentiation of effector cells. Malignant disease in the otherwise immunocompetent host could escape immune control through failure to recognize tumor cells as foreign or through lack of the required "second signal".

Previous work suggested that Interleukin 2 may be one of the second signals stimulating immune cells. Failure of Interleukin 2 production or action may allow malignant cells to escape the immune system. Therefore, an attractive method of immunotherapy would be to increase the positive immunoregulatory action of IL2 *in vivo*. Unfortunately, methods for modulating Interleukin action *in vivo* are not available. Therefore, removing lymphocytes from tumor-bearing mice, culturing them with exogenous Interleukin 2 and, subsequently, reinfusing the programed cells into tumor-bearing mice was explored as a model of a possible immunotherapeutic technique.

Spleen cells from tumor-bearing mice contain populations of precursor cells reactive to autologous tumor. Culture of these cells with exogenous Interleukin 2 generated a population of helper lymphocytes able to recruit host anti-tumor activity. Culture with IL2 also generated two populations of lymphocytes directly cytotoxic to tumor cells. One of the populations of cytotoxic lymphocytes, generated by culture with Interleukin 2 and autologous tumor, was relatively specific to the sensitizing tumor. These are probably "classical" cytotoxic T lymphocytes. The second population of cytotoxic cells, generated by culture with Interleukin 2 alone, demonstrated a broader spectrum of anti-tumor reactivity. Identifying the origin of the cell responsible for this non-specific activity has proven to be difficult. The broad spectrum of activity, the lack of requirement for antigen sensitization, and the lack of H2 restriction are appropriate for "natural killer" cells; whereas, the time course of activation and the surface marker phenotype are appropriate for "classical" cytotoxic lymphocytes. The precursors of both cytolytic cell populations, described above, are significantly increased in tumor-bearing animals. This suggests that tumor recognition occurs in tumor-bearing animals but that the "second signal" required for proliferation and differentiation is not present or not received. Interleukin 2 can provide this signal at least *in vitro*. Culture of peripheral

blood cells from ovarian cancer patients with either human IL2 or murine IL2 generated cytotoxic lymphocytes which were active against autologous tumor.

In the immunotherapy of murine tumors, the cytotoxic lymphocyte containing populations were most effective if given shortly after injection of the tumor. The response was dose related. Repeated injections were more effective than single injections. The cultured cells homed poorly to the tumor, therefore injection directly into the tumor site was more effective than intravenous administration. Therapy with cytotoxic lymphocytes was synergistic with surgical therapy of CaD2 tumors.

Therapy with cytotoxic lymphocyte containing populations consistently improved the survival of mice with intraperitoneal P815 tumors. Despite the improved survival of mice following therapy, there were few long term survivors. Therapy with cytotoxic lymphocyte containing populations cured some mice with subcutaneous P815 tumors. The mice that died of the tumor did not demonstrate an improvement in survival times compared to untreated mice. Mice cured of the P815 tumor by treatment with cytotoxic lymphocyte containing preparations remain tumor-immune.

There were no significant detrimental side effects of therapy with cytotoxic lymphocytes.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Verner Paetkau, for advice and patience while allowing me to go my own way. I would like to thank all the people with whom I worked, especially Stan Gerhart, Jennifer Shaw, and Barry Caplan, for assistance, materials and listening to my ideas. Special thanks to Vicky Monticone, Cliff Gibbs, and Thealzel Lee for technical assistance and to P. d O'Brenan and my sister, Cathy Langner, for assistance in preparing this thesis. Financial support was from the Medical Research Council and the Alberta Heritage Savings Trust Fund, Applied Cancer Research Program. I would also like to thank my present colleagues for making me realize it was all worth it.

ABBREVIATIONS

B cells	Bursa-derived cells
BSA	Bovine serum albumin
C	Complement
Chymo	Chymotrypsinogen A
CL	Cytotoxic T lymphocyte
CLP	Cytotoxic T lymphocyte precursor
CLPa	Activated cytotoxic T lymphocyte precursor
Con A	Concanavalin A, a mitogen
Cr	Chromium
CSF	Colony Stimulating Factor
CSF-GM	Colony Stimulating Factor-Granulocyte-Monocyte
Cyto	Cytochrome C
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
GVH	Graft versus host
H2	Major histocompatibility complex of the mouse
HLA	Major histocompatibility complex of the human
HY	Male transplantation antigen
Ia	I region associated antigen
IL 1,2,3	Interleukins 1,2, and 3
i.p.	Intraperitoneal
IR	Immune response
IUdR	Iododeoxyuridine
i.v.	Intravenous
KA	Killing activity
LAF	Lymphocyte Activating Factor - IL 1
MIs	M locus, minor histocompatibility antigen
MW	Molecular weight
NK	Natural killer cells
NMS	Normal mouse serum

Ova	Ovalbumin
PMA	Phorbol myristate acetate
S	Stimulator cell in CL generation
s.c.	Subcutaneous
SDS	Sodium dodecyl sulfate
T	Thymus-derived cell
Ta	Activated T cell
Thy 1	Surface marker of T lineage in the mouse
UV	Ultraviolet irradiation

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1. INTRODUCTION

1.1 THE IMMUNE SYSTEM

1.1.1 BACKGROUND

During evolution, the immune system developed for the purpose of clearing foreign pathogens. Many disease states result from aberrations of the immune system. To prevent these diseases, the immune system must respond effectively, resulting in the rapid clearance of foreign challenges. Following antigen clearance, the immune system must return to a baseline state to prevent immunoproliferative disorders. Fine specificity is also required to ensure that host damage does not occur through reactions directed against "self"-antigens. Effective antigen clearance without host damage requires a complex network of control elements.

1.1.2 CONTROL ELEMENTS

Although the control elements generally ensure that the immune system eliminates pathogens without host damage, the system occasionally fails. Effector mechanisms may be inadequate or so completely damped by control systems that persistent or overwhelming infections occur. Too much suppression of effector mechanisms may result in progressive malignant disease. Immunodeficiency disease could result either from genetically abnormal effector mechanisms or from excessive suppression of the effector cells by control systems. Conversely, stimulatory elements of the control system may become autonomous resulting in autoimmune or immunoproliferative disease.

1.1.3 EFFECTOR CELLS OF THE IMMUNE SYSTEM

Historically the immune system has been divided into humoral and cellular effectors by the ability to passively transfer immunity by serum or cells, respectively. The two specific effector arms of the immune system, humoral and cellular, are mediated by bone marrow-derived (B) and thymus-derived (T) cells, respectively. The specific effector cells of cellular immunity are cytotoxic T lymphocytes (CL) and delayed type hypersensitivity (DTH) mediators. CL act by binding to cells and delivering a "kill" signal. DTH mediators

release lymphokines which attract other cells to the site. These cells are then activated to clear the foreign pathogens. The humoral arm is mediated by antibodies secreted by plasma cells. These effector systems are under the control of a network of stimulatory (helper) and inhibitory (suppressor) T cells.

For the generation of effective immune responses, foreign antigens must first be identified as non-"self". The antigens must then be presented by antigen-presenting cells, usually macrophages. This initiates a cascade of reactions involving T cells and B cells which results in the proliferation and maturation of effector cells. These cells then mediate the rapid clearance of the foreign pathogen.

1.1.4 CELLS MODULATING THE IMMUNE SYSTEM

The need for interaction between T and B cells in generating antibody responses is well established (160). The requirement for T cell-T cell collaboration in cell mediated immune responses was first demonstrated in the development of graft versus host (GVH) responses (38) and, subsequently, in the development of specific CL responses (9). Helper, suppressor, and contrasuppressor cells modulate the immune system (123, 160).

1.1.5 MAJOR HISTOCOMPATIBILITY LOCUS

The observation that tumors and skin grafts were rapidly rejected led to the discovery of the major histocompatibility antigens. Allogeneic (the same species but with different genetic background at the major histocompatibility locus) skin grafts were rejected more rapidly than xenogeneic (other species) skin grafts or semi-syngeneic (the same species with the same major histocompatiblity locus but with a different genetic background elsewhere) skin grafts. The foreign (non-"self") histocompatibility antigens present on the surface of allogeneic cells resulted in the generation of vigorous immune responses. Although allogeneic histocompatibility antigens can lead to the rejection of foreign tissue, this is not the function of the major histocompatibility locus. The recognition of "self" histocompatibility antigens is required for communication between immune cells, for the recognition of foreign pathogens such as viruses and for the prevention of damage to autologous ("self") cells. The highly polymorphic nature of the major histocompatibility system may prevent viral or pathogen induced diseases from

escaping the immune system.

The cell surface products of the highly polymorphic major histocompatibility locus can be divided into classes based on their role in T lymphocyte responses. Class I antigens are normally involved in the recognition of foreign antigens expressed on virus-infected or pathogen-infected cells. Class II antigens are normally involved in cell-cell communication (365). In the mouse, class I antigens are designated H2-K and H2-D. In the human, they are designated HLA-A, HLA-B, and HLA-C. The class II antigens are called Ia-A and Ia-E in the mouse and HLA-DR, HLA-MT, and HLA-SB in the human. The class II antigens of both species are often collectively referred to as "Ia". Each of the genes of the major histocompatibility locus has several alleles which code for cell surface proteins. These proteins have been identified by reactions with specific antibodies or with specific T cells. Class I antigens are expressed on almost all cell populations while class II antigens are expressed on a much more limited cell population including macrophages, B cells, activated T cells, and some epithelial cells (240).

Class I antigens, in conjunction with antigens induced by virus or other pathogens, are recognized by CL and DTH mediators and their precursors. This requirement for the recognition of normal "self" proteins in association with proteins induced by foreign pathogens results in the "restriction" of immune responses (365). Responses to foreign antigens are restricted by the need for the presence of "self" proteins for the development and the expression of the immune response. This restriction was first noted by the inability of CL to kill allogeneic cells which expressed an antigen which would result in the killing of "self" cells (365).

Recognition of "self" class II antigens seems to be involved in most T-macrophage and T-B cell interactions. Helper T cells generally react with class II antigens rather than with class I antigens. Antigen presentation by macrophages is more efficient with "self" class II antigens than with allogeneic class II antigens (327). Antigens presented with class II antigens elicit more vigorous immune responses than do soluble antigens alone (327). The presence of class II antigens on the surface of activated T cells suggests that these antigens may also be involved in T-T collaboration (240).

Many other antigens are capable of inducing graft rejection. These less active antigens are designated minor histocompatibility antigens. They include male (HY) antigens,

M locus (Mls) antigens, tumor antigens, and differentiation antigens. The genes for these antigens may be located at sites distant from the major histocompatibility locus. For example, the mouse Mls locus is on chromosome 1 while the major histocompatibility complex is on chromosome 17.

1.1.6 ANTIGENIC MARKERS ON T CELLS

Both class-specific and differentiation-specific antigens have been identified on T cells. Monoclonal antibodies and hetero-antisera have been developed that bind to these antigens. Occasionally, these surface proteins have unique characteristics such as the ability to bind xenogeneic erythrocytes. For example, the presence of the Thy 1 antigen on mouse T cells and the sheep erythrocyte receptor on human T cells allows the separation of T cells from other cells of the immune system. The presence of distinct cell surface markers has aided in the identification, characterization and purification of several separate T cell populations (39, 172).

Murine helper T lymphocytes and DTH mediators were thought to bind antibodies against the Ly 1 surface antigen but not to bind antibodies against the Ly 2 and Ly 3 antigens. Usually, suppressor T lymphocytes and effector CL bind antibodies against the Ly 2 and Ly 3 antigens but not antibodies against the Ly 1 antigen. Precursor T cells of both groups usually express all three surface antigens (39). Antibodies to these cell surface markers allow both positive and negative selection of the various cell types and, therefore, studies of more homogenous cell populations. Unfortunately, in certain mouse strains or with certain antigenic stimuli, the designation of cell type by use of the aforementioned pattern of differentiation antigens does not hold (8, 140). These exceptions prevent designation of cell function on the basis of surface phenotype alone.

Similar differentiation markers, identified by monoclonal antibodies, are present on the surface of human T cells. Originally, human helper T cells were thought to bind the OKT 4 monoclonal antibody while CL and suppressor T cells were thought to bind the OKT 8 antibody (172). Subsequently, it appeared that cells responding to class I major histocompatibility antigens bound the OKT 8 antibody, while those responding to the class II histocompatibility antigens bound the OKT 4 antibody (240). It now appears that this is not always correct, as all possible combinations of OKT 4 and OKT 8 binding occur with

clones of helper T cells and CL reacting with class I or class II major histocompatibility antigens (298).

1.1.7 OTHER CELLS INVOLVED IN IMMUNE RESPONSES

In addition to the specific effector and modulatory T and B cells described above, many other cell types, including natural killer (NK) cells, macrophages, and neutrophils, are involved in both specific and non-specific clearance of foreign antigens. NK cells seem to react with a common structure or structural change expressed by many malignant cells. They are particularily reactive with malignant cells of lymphoid origin. Since NK cells do not need priming to be effective, it has been hypothesized that they may deal with very early malignant changes or with very early viral infections. More recently, it has been hypothesized that they may play a regulatory role in the differentiation of hematopoietic cells. Macrophages play several roles. They are involved in antigen binding, processing, and presentation. In addition, they release many active monokines which activate T cells, other macrophages, neutrophils, and several other cell types. Activated macrophages may also play a direct role in antigen clearance by phagocytosis and destruction of foreign pathogens. Neutrophils are attracted to sites of foreign antigens. They also release active factors and phagocytose several types of foreign bacteria.

1.1.8 REPLACEMENT OF IMMUNE CELLS BY NON-SPECIFIC SOLUBLE FACTORS

Non-specific soluble factors can replace most, but not all, of the functions of macrophages and helper T cells (205,213,232,246,272,283,327). Despite being non-specific in action, these soluble factors must act in such a way as to maintain the specificity of the immune system. Otherwise autoimmune disease would result. Probably, the specificity of the system is maintained by the action of the non-specific factors on antigen-stimulated cells and by the action of the non-specific factors over short distances.

1.2 MACROPHAGE-T CELL INTERACTION

Macrophages are necessary for the initiation of CL activation by foreign antigens (327). Macrophages effectively present foreign antigens to T cells and also release soluble factors necessary for T cell activation (327). In turn, activated T cells in association with foreign antigens are required for the stimulation of the macrophages participating in CL induction (211,216). Both the macrophage and the T cell can be replaced by non-specific soluble factors (183,216). Colony stimulating factor, granulocyte-monocyte (CSF-GM), produced by activated T cells, can replace the need for T cells in macrophage activation. Interleukin 1 (IL1, formerly designated Lymphocyte Activation Factor, LAF, 1), produced by CSF-GM activated macrophages, can replace the need for macrophages in T cell activation.

1.2.1 IMMUNE RESPONSE GENES

Immune response (IR) genes play a controlling role in some murine immune responses. In the mouse, the IR genes have been mapped to the I region of the major histocompatibility locus. The protein products of the two I region loci, I-A and I-E, are the class II major histocompatibility antigens Ia-A and Ia-E (272,327). The activation of macrophages by T cells and the activation of helper T cells by macrophages normally requires identity between the interacting cell types at the class II major histocompatibility locus (80,271,272). Either or both cell types can be replaced by Ia non-restricted soluble factors (183,216). T cell interaction with Ia on macrophages may be necessary for the generation of helper T cells. Otherwise, the generation of CL may be Ia or macrophage independent (59,183).

Immune response genes can determine the ability of macrophages, T cells and B cells to collaborate to produce effective responses against some antigens. The genetic inability to respond to some antigens may reside in either the macrophage or the T cell (327). In the circumstance where the macrophage is responsible for the lack of immune responsiveness, the lack of responsiveness may be due to a lack of production of a soluble factor (271,272,327), perhaps IL1.

The cell surface products of the human HLA-DR, HLA-MT, and possibly the HLA-SB loci share significant structural, antigenic and functional homology with Ia proteins in the

mouse (240). These human proteins seem to play the same role in cell-cell communication that their murine homologues do. Human IR gene restricted responses may also be governed by genes located in this area.

1.2.2 Ia IN INTERLEUKIN PRODUCTION

Since the need for Ia identity between T cells and macrophages in CL generation can be replaced by Ia non-restricted soluble factors (85,216), Ia recognition and binding may directly activate the cells. Instead, Ia recognition and binding may function to keep the cells in close proximity. This close proximity may be necessary for the normal *in vivo* action of the soluble factors. Macrophage activating factors, CSF-GM, and the T cell activating factor, IL1, probably are short range communication factors limited by adsorption (107) or degradation (85). Interleukin production, CL generation and lymphocyte proliferation *in vitro* can be blocked by some anti-Ia antibodies (113,303). These antibodies may act by preventing the formation of the microenvironment needed for factor production and effect. Exogenous IL1 or CSF-GM can abrogate the need for Ia binding to keep the macrophage and T cell in close proximity (83,113,183,285,292,293,294). Mitogens, such as Con A, may remove the need for Ia identity by non-specifically binding cells together, thus establishing the needed microenvironment (281).

The main role for Ia recognition by T cells seems to be the production of helper factors by helper T cells. One of these helper T cell products, designated Interleukin 2 (IL2, 1), is responsible for the proliferation of activated CL.

1.2.3 CHARACTERIZATION OF SOLUBLE FACTORS INVOLVED IN MACROPHAGE-T CELL INTERACTION

CSF-GM produced by T cells has a molecular weight of 25-35,000 (216). IL1 produced by macrophages has a molecular weight of 12-18,000 (213). IL1 purified to apparent homogeneity is active at 10^{-10} to 10^{-11} M (214). Both factors are antigen non-specific and H2 non-restricted in action.

1.2.4 CSF-GM IN INTERLEUKIN PRODUCTION

Normally, macrophages are activated by a T cell separate from the IL2 producing helper T cell (183). Once the macrophage is activated, culture with CSF-GM and antigen is sufficient for the production of IL1 (216). The IL2 producing cell can produce CSF-GM after activation by IL1 (216). Therefore, once the system is activated, it can become self-perpetuating. As long as antigen is present, the macrophage can produce IL1 which can stimulate the helper T cell to produce CSF-GM. This CSF-GM then stimulates the activated macrophage to produce more IL1; thus generating a self-perpetuating system. This auto-stimulatory network may be responsible for the continuation and amplification of IL2 production by helper T cells.

1.2.5 OTHER MACROPHAGE FUNCTIONS

In addition to their role in IL1 production, macrophages play other roles in antigen clearance. Their efficient phagocytosis of foreign antigens along with their antigen-processing, and antigen-presenting activities, make macrophages integral to many immune responses. For example, macrophages cannot be totally replaced by soluble factors in responses to some mitogens (233) or in colony formation (Lederman and Mills, unpublished data). Macrophages provide efficient presentation of antigens such as albumin (327) and mitogens such as Con A (205). Activated macrophages may play a direct role in tumor clearance by the phagocytosis and destruction of tumor cells.

1.3 T CELL-T CELL COLLABORATION

As mentioned previously, T cell-T cell collaboration is necessary for the generation of both CL and DTH mediators (160). The overall effector response depends on the balance of T help, T suppression and possibly T contrasuppression (123, 160). Many of the functions of helper T cells can be replaced by the myriad of helper factors produced by the helper T cells. One helper T cell clone produces at least three different helper factors: IL2, CSF-GM, and interferon (251). The same clone produces activities assayed as B cell stimulating factor, macrophage recruiting factor, interleukin 3, Ia inducing activity, Fc receptor enhancing activity, and macrophage activating factor. Whether all these activities are mediated by the three characterized molecules listed above is not clear (251).

Helper cells, in most assays, can be replaced by one or another of their helper factors. For the development of CL from precursors, helper T cells can be replaced by IL2, interferon and a group of less well defined terminal differentiation factors (85,122,254,256,257,269,283). Cell-cell contact or Ia recognition may not be necessary for activation by helper T cells.

1.3.1 INTERLEUKIN 2 IN T CELL-T CELL COMMUNICATION

One of the factors involved in T cell-T cell collaboration is IL2 (283). The term, IL2, refers to a lymphokine previously designated as co-stimulator, T cell growth factor, thymocyte mitogenic factor, thymocyte stimulating factor, or secondary cytotoxic T cell inducing factor (1). IL2 is defined by the unique ability to maintain activated T cells in continuous proliferation.

The presence of an assortment of factors in supernatants derived from activated spleen or peripheral blood cells has made it very difficult to determine which factor is responsible for a given biological effect. The use of impure or only partially purified preparations has not alleviated the problem. The difficulty in designating the activity of a given factor is further complicated by the ability of one lymphokine to activate cells to produce other immunoregulatory lymphokines. Since the crude biological assays used can by activated by several mechanisms, it is difficult to dissect out the activity of any one factor. The identification of specific physiological roles for each factor will require:
1). pure factors
2). knowlege of the factor's position in activation cascades and
3). specific antibodies or inhibitors for each of the factors in the activation cascade.
Further studies with purified IL2 and with specific inhibitory antibodies may result in some of these activities being attributed to other factors or being an end result of a cascade of which IL2 is only a part.

1.3.1.1 CHACTERIZATION OF IL2

Murine IL2 is an acidic glycoprotein with an apparent native molecular weight of 31,000 (284). It is normally produced by a Thy 1^+, Lyt $1^+ 2^- 5^+ 6^- 7^-$, Qa 1^- helper T cell (203,285). Since murine IL2 moves during electrophoresis on SDS polyacrilamide gels or on chromatography on a sephacryl S-300 column equilibrated with SDS with an apparent

molecular weight of 16,000, murine IL2 is probably a dimer (40,85). It is active at a concentration of 3×10^{-12} M or lower (40,85). Murine IL2 has little, if any, activity in maintaining continuous proliferation in human systems (110).

Human, gibbon, and rat IL2 are all proteins with a molecular weight of approximately 15,000 (106,2,53). IL2 from these species maintains continuous proliferation of CL in murine as well as homologous systems.

Gel filtration chromatography of IL2 produced from normal cells gives a broad activity peak. This broad peak may occur because of differential glycosylation of IL2 during synthesis (106,284). The wide range of isoelectric points of IL2 also suggests glycosylation differences. Neuraminidase treatment can decrease the charge heterogeneity of IL2 (106). IL2 produced by the human cell line, Jurkat, does not appear to be glycosylated. Jurkat IL2 has a sharper gel chromatography profile and less charge heterogeneity (106).

The cDNA for Jurkat IL2 has been identified, isolated and sequenced from a cDNA library prepared with partially purified Jurkat mRNA (313). A proposed amino acid sequence of 153 amino acids would encode a molecule of 17,632 daltons. If a 20-amino acid hydrophobic tail was cleaved during secretion, the molecular weight would be 15,420 (313) which is the molecular weight reported for Jurkat IL2 (106). The proposed amino acid sequence predicts a neutral isoelectric point. There are no sites for n-glycosylation present. The IL2 cDNA has been transfected into a monkey cell line which then produces an IL2 indistinguishable from Jurkat IL2 confirming that the cDNA is that for Jurkat IL2. There appears to be only one copy of the IL2 gene in the haploid human genome. The gene appears to be located on chromosome 4.

1.3.1.2 PRODUCTION OF IL2

Production of IL2 from unprimed cells requires interaction of a helper T cell with either antigen or mitogen (232) and Ia^+ macrophages or their product, IL 1 (183,184,205,292,293,304). With antigen-primed T cells, antigen alone may be sufficient for the induction of IL2 production (33,341).

IL2 production occurs maximally during the first 18-36 hours of culture (283). Thereafter, the amount of IL2 present in the supernatants decreases due to adsorption to cells (234) and due to degradation (85). The effect of culture-induced suppressor cells

(131) and factors (170) may decrease the measurable IL2 activity. Cellular proliferation is not necessary for IL2 production (68). RNA synthesis and protein synthesis are both necessary for IL2 production and release (68).

1.3.1.3 CELL RESPONSIBLE FOR IL2 PRODUCTION

Because both macrophages and T cells are necessary for IL2 production, it was initially difficult to determine which cell produced IL2. Production of IL2 from macrophage-depleted T cells in the presence of the macrophage product, IL 1, indicated that the producing cell was likely to be a T cell (183,285). The production of IL2 by helper T cells has been confirmed by IL2 production from murine T cell lines (82,105,114), from human T cell lines (105), from gibbon T cell lines (253), and from murine T cell hybridomas (136,241).

The surface antigen phenotype of the murine helper T cell producing IL2 can vary with the antigenic stimulus (8,140). In most activating situations, the helper T cell reacts with class II proteins. These helper cells are Ly 1^+ 2^-. Helper T cells producing IL2 in response to class I differences are Ly I^- 2^+ (8,140).

The frequency of Ly I^+ IL2-secreting helper cells reactive to specific Mls antigens is between 1 in every 30 to 300 T cells (203). Precursors responsive to the H2 antigens are 5-10 fold less frequent. Clones of helper T cells produce 30 times more IL2 than expected from predictions made with bulk cultures. This suggests that IL2 production in bulk cultures is tightly regulated (203).

In the human system, an OKT 4^+8^- T cell produces IL2 in response to allogeneic class I antigens. An OKT 4^-8^+ T cell secretes IL2 in response to allogeneic class II antigens (239,240).

1.3.1.4 MECHANISM OF ACTION OF IL1 IN IL2 PRODUCTION

IL 1 binds specifically to the IL2 producing cell (107). IL1 receptors do not appear to be present on the surface of unstimulated T cells. Foreign antigen, presented by Ia-identical macrophages, results in the expression of IL1 receptors by helper T cells (94,107,240,261). Culture with mitogen, in the absence of Ia-identical macrophages, can result in the expression of IL1 receptors. Although the need for Ia-identical macrophages can be abrogated by culturing cells with mitogen (240), the physiologic stimulus for IL1

receptor expression is foreign antigen presented on Ia-identical macrophages (94,107,240,261).

Human, gibbon, and murine helper T cell lines producing IL2 have been identified. Some of these lines secrete IL2 constitutively. Others secrete IL2 following culture with antigen alone. Some cell lines secrete IL2 only after culture with both IL 1 and antigen (94,107,253). The cell lines, requiring culture with both exogenous antigen and IL 1 to induce IL2 release, may represent the normal *in vivo* situation. The lines releasing IL2 constitutively or the lines requiring culture with antigen to induce IL2 release may represent abnormal cells. These abnormal cells may have been induced in culture or may have been induced by malignant transformation of the original cells (94, 107).

1.4 CL GENERATION

Generation of CL requires at least two steps; the first being the development of IL2 receptors and the second being proliferation in response to IL2 (85,99). A third step, the production and response to immune interferon, may also be required (84, 106). Other terminal differentiation factors may also be involved (122,256,257,269,342).

Cells lacking IL2 receptors do not proliferate in response to IL2. Proliferation in response to IL2 can be related to the presence of IL2 receptors and to the concentration of IL2 present (259,283). The IL2-driven proliferation of T cells is responsible for many of the activities attributed to IL2 (99, 110,215,275). Although cells of the CL lineage appear to be particularily responsive to IL2 (99, 110), other T cell subclasses can respond to IL2 under appropriate conditions.

1.4.1 IL2 RECEPTORS

Murine spleen cells express, on the average, less than 60 IL2 receptors on their surface (259). Activation with Con A or alloantigen increases the receptor number to 3,000 to 9,000 per cell (259). CL clones, continuously proliferating in the presence of IL2, express up to 15,000 IL2-specific receptors per cell. These receptors bind IL2 tightly having a dissociation constant of about 1×10^{-11} M (259). There is only one class of IL2 receptors present on the surface of T cells.

Human peripheral blood cells demonstrate a somewhat different pattern. They express, on average, 200 receptors per resting T cell. Activation by antigen increases the number of receptors to 11,000 per cell. Human IL2 receptors have a dissociation constant of $5\text{-}7 \times 10^{-12}$M (259). The presence of an average of 200 receptors per cell in peripheral blood could represent a low number of receptors on each cell or, more likely, it represents a higher number of receptors on a small population of activated cells (259). This activated cell may represent the cells in human peripheral blood which develop into NK-like cells following culture with IL2 alone (163,169,229).

IL2 binds to two proteins in solubilized membrane preparations. These proteins have molecular weights of 40,000 and 60,000. The 40,000 dalton protein seems to be a precursor of the 60,000 dalton protein (K. Smith, personal communication). The IL2 receptor does not bind and is not competitively inhibited by LAF (IL 1), fibroblast and immune interferon, CSF, epidermal growth factor, neuronal growth factor, fibroblast growth factor, multiplication stimulating factor, erythropoeitin, or insulin (259).

Elution of IL2 from IL2 receptors occurs following incubation with glutaraldehyde, glycine, or acetic acid (85,259,291). Heating can also release IL2 from the IL2 receptor (85). Internalization and degradation of the IL2-IL2 receptor complex occurs following incubation at 37° C. Internalization of the IL2-IL2 receptor complex may be necessary for cellular activation or may be necessary for removing IL2 from the cell surface.

Monoclonal antibodies to IL2 receptors have been produced (210,230,324). The anti-Tac antibody reacts directly with the IL2 receptor (210,324). The anti-Tac antibody prevents binding of IL2 to its receptor (324). This antibody precipitates proteins with molecular weights of 40,000 and 60,000 from solubilized membranes of activated T cells. These proteins have the same size as those which bind IL2. A second antibody, blocking IL2 activity following binding to cells, has been identified (230). Although this antibody prevents activation by IL2, it does not block binding of IL2 to its receptor. This antibody may bind to the IL2 receptor at an epitope different from the IL2 binding site (230). Alternatively, this antibody may block activation by a mechanism unrelated to the IL2 receptor.

1.4.1.1 INDUCTION AND EXPRESSION OF IL2 RECEPTORS

Culture with antigen alone results in expression of the IL2 receptor by cytolytic T cell precursors (CLP) (59,180,181). This is in contrast to the expression of the IL1 receptor by helper T cells. IL1 receptor expression requires culture with both antigen and Ia-identical macrophages. Following stimulation with antigen, the expression of IL2 receptor occurs rapidly (less than five hours). Protein synthesis is required (182). Expression of IL2 receptors does not require cellular division and does not require capping of mitogen receptors (182). Antigen must be on metabolically active cells to result in the expression of IL2 receptors (182). Interaction with macrophages and helper T cells is not required (59,180,181).

T cells stimulated by allogeneic cells can be separated from the original stimulating cells. Addition of exogeneous IL2 to cultures of single cell precursors is sufficient to result in the development of CL active against the original stimulating antigen (202). Therefore, once the lymphocyte is stimulated by antigen, and IL2 receptors are expressed on the cell surface, antigen is no longer needed to maintain the specificity or to maintain the proliferation of the alloantigen-stimulated cells. The only requirement for continued growth seems to be IL2 or a factor contained in partially purified IL2 preparations (202). The continuous proliferation of alloantigen-specific cells in IL2-containing preparations suggests that once IL2 receptors are expressed on the surface of the alloantigen-stimulated CL, the receptors remain on the surface of subsequent generations of cells (99). The specificity is determined by the initial sensitizing antigen and is maintained in cultures with IL2 alone. The CL may lose specificity following long term culture in IL2 (117).

1.4.1.2 DISSOCIATION OF INDUCTION OF IL2 RECEPTORS AND IL2 RELEASE

Several systems wherein antigen stimulation is not sufficient for generation of CL have been identified (103,143,176,255,274,311,340,341). In these systems, the addition of exogenous IL2 generates significant numbers of CL. Incubation with IL1 also results in the generation of specific CL, suggesting that the block probably occurs at, or before, the production of IL1 (8). The simplest explanation is that incubation with these antigens results in the expression of receptors for IL2 without resulting in the generation of IL1 and thus IL2. Therefore the density or configuration of the antigens that induce IL2

receptors may be different from the density or configuration of antigens that induce IL2 synthesis and release (234).

The presentation of antigen on metabolically inactive cells leads to the expression of IL2 receptors without the generation of CL (211,283,305). Culture with exogenous IL2 results in the generation of CL. Similarily, incubation with inhibitors including pyrilamine, a specific histamine H1 antagonist (305), corticosteroids (103), cyclosporin A (141), or prostaglandin E (255) all result in the expression of IL2 receptors without the generation of CL. Culture with exogenous IL2 results in the generation of CL. Similar expression of IL2 receptors without the generation of IL2 may occur in cultures with syngeneic tumor cells (206). Addition of exogenous IL2 also increases the generation of CL active against the syngeneic tumor cells (206).

1.4.2 IL2 IN PROLIFERATION AND DIFFERENTIATION OF CL PRECURSORS

Partially purified IL2 is sufficient to drive antigen-activated or mitogen-activated CL precursor cells to clones of effector CL (305,315). Precursor cells responsive to specific alloantigen and exogenous IL2 occur at a frequency of about three per thousand spleen cells. When IL2 concentrations are high, only about one-third of the cytotoxic clones are antigen-specific (305,315). The proliferation and differentiation of precursor cells to effector CL was attributed to the presence of IL2 (206,284), but the IL2 preparation used was known to also contain immune interferon (vide infra). When cells were cultured in interferon-free preparations, the frequency of responding clones decreased to about one third of that occuring when exogenous interferon was present in the cultures (H.S Teh, personal communication). This suggests that exogenous IL2 is sufficient for the maturation of some CL precursor cells but that both exogenous IL2 and exogenous interferon are needed for the maturation of some precursor cells. An effect of interferon, even in the cultures with the interferon-free exogenous IL2, could not be ruled out. CL can produce interferon upon specific antigen stimulus (168,217). Therefore, the CL precursors, stimulated with antigen and cultured with exogenous IL2, may have been able to produce sufficient interferon to result in the generation of effective CL. The possible role of other terminal differentiation factors (256,257,342) which might have been contaminating the IL2 preparations or might have been released

from the CL precursors was not studied. Although culture with interferon-free IL2 and antigen is sufficient to generate CL, a role for endogenous interferon cannot be ruled out.

1.4.3 SPECIFICITY OF CL INDUCTION BY IL2

CL produced by culture with a given antigen are normally specific to the sensitizing antigen. This specificity occurs despite the non-specific action of the factors, IL1, IL2, and interferon. Several mechanisms are involved in maintaining specificity. Firstly, the release of the non-specific factors requires an antigenic stimulus. Secondly, each step in CL activation can be performed by a non-specific soluble factor with the **exception** of antigen-driven acquisition of IL1 and IL2 receptors. Therefore, antigen-driven production of the factors and antigen-driven acquisition of IL1 and IL2 receptors help to maintain the specificity of the CL.

Given that specific receptors exist for IL1 and IL2 and that binding of the respective factor to its receptor results in loss of the factor (25,80,175,234), these stimulatory factors probably act over a very short distance. Furthermore, the presence of an IL2 inhibitor in the sera of mice (135) and the rapid clearance of IL2 from the sera of normal mice (236) ensures that IL2 has a very short range of action. This short range of action may prevent the activation of "bystander cells".

Culture with high levels of exogenous IL2 stimulates "bystander cells" and clones of cells with polyspecific activity (99,206,229,306). High endogenous levels of IL2 may also result in the breakdown of specificity. This may be one of the causes of autoimmune disease.

1.5 CONTINUOUS PROLIFERATION OF T CELLS

The continuous proliferation of activated T cells in the presence of IL2 allows the establishment of cell lines of several T cell subsets. These lines include CL activated by alloantigens (99), syngeneic tumor antigens (99,110,236), soluble protein antigens (300), and viral antigens (99). Helper T cell lines have been established (93,116,117,281,282,314,345) as have suppressor T cell lines (16,144,260). T cell lines mediating DTH activity have also been established (196,319) as have lines mediating both DTH and CL activities (67).

Cell lines initially sensitized with antigen tend to have the characteristics of specific CL (24,99). Cells cultured in IL2 without added antigen tend to develop the characteristics of NK cells (66,277,286,302,333). Although the continuous presence of stimulating antigen is not strictly required for the proliferation of some CL lines (99), antigen may aid in maintaining the specficity of the cell line and in the initial cloning of the cell line (117). Helper cell lines are most easily established with the continuous presence of antigen (281,345).

Many of these cell lines and clones are polyfunctional in their activities. Some secrete several different helper factors (94,116,251,281,345). Some lines which release interferon also mediate CL activity (168,217) or DTH activity (196). Some cell lines mediate both CL and DTH activity (67). These polyfunctional activities suggest that effector T cells drawn to sites of foreign antigens may possess several mechanisms for increasing antigen clearance and thus improving host survival.

The continued proliferation of activated T cells in the presence of IL2 has aided in the characterization of monoclonal populations of T cells. This has resulted in a much improved understanding of proliferation, antigen specificity, and inter-cellular communication. Since the cell lines and monoclonal populations have been established with, at best, partially purified IL2, the role of the many other factors known to be present in supernatants produced by activated T cells is not clear (251). Other important factors may include immune interferon (168,217), interleukin 3 (IL3) (85,134,152,153,175) and terminal differentiation factors (91,122,255,257).

1.6 OTHER FACTORS INVOLVED IN CL GENERATION

1.6.1 INTERFERON

The term, interferon, is used in this thesis to refer to the form of interferon released by lymphocytes and active on lymphocytes. This type of interferon is also referred to as immune interferon. Although this form of interferon does express activity against viruses, its primary action seems to be as an immunomodulatory lymphokine. Appropriate antigen stimulation or mitogen stimulation of lines of cells, mediating CL activity (168,217), DTH activity (196), or NK activity (133), results in immune interferon

production. Because of the production of immune interferon by the CL responding to IL2, it will be difficult to determine the exact role of each of these two factors in CL generation.

1.6.1.1 PRODUCTION OF IMMUNE INTERFERON

Stimuli which increase IL2 production also increase immune interferon production (84,225). IL2 may increase interferon production but is not an essential prerequisite for interferon production. Culture with ultraviolet-treated virus-infected cells results in interferon release without measurable IL2 release (225). Antigen, Ia-identical macrophages, Ly I$^+$ T cells, and Ly 2$^+$ T cells are normally required for immune interferon production (320). Partially purified IL2 can replace the requirement for macrophages and the Lyt 1$^+$ T cells in the generation of immune interferon (320). In most circumstances, interferon is, therefore, produced by a Ly 2$^+$ T cell (320).

1.6.1.2 INTERFERON MAY INDUCE IL2 RECEPTORS

Because the induction of CL can be abrogated by removal of either IL2 or immune interferon, the two factors probably do not act in a sequential manner (85,109,166,175). The effect of exogenous IL2 and interferon on CL generation is more than additive, suggesting a synergism between the two lymphokines (175).

Culture with exogenous interferon increases the specific adsorption of IL2 by spleen cells (85,175). Antigen is not required for this action of interferon. Culture with interferon increases either the number of IL2 receptors, the affinity of the IL2 receptors, or the internalization of the IL2-IL2 receptor complexes (85,175). Interferon, produced by the IL2 responsive cells, may have an autosensitizing effect for IL2. This may replace the need for antigen-driven generation of IL2 receptors. This interferon-driven generation of receptors for IL2 (85,169,320) may explain the generation of non-specific CL or the generation of non-specific NK-like cells. These non-specific cells occur most frequently in cultures with high concentrations of exogenous IL2 (206,306).

1.6.1.3 INTERFERON INDUCES Ia

Interferon is necessary for the maintenance of Ia on the surface of macrophages (D. Beller, personal communication). Since Ia normally plays a role in IL2 production (113,303), interferon may also have an indirect effect on IL2 production. The normal

endogenous concentration of interferon is sufficient to maintain optimal number of Ia antigens on macrophages. Therefore, the function of the modulation of Ia antigens by interferon is not clear.

1.6.2 INTERLEUKIN 3

IL3 was initially identified by its ability to induce 20 alpha-hydroxysteroid dehydrogenase activity in spleen cells from nude mice and in thymocytes from normal mice (152). IL3 may also play a role in establishing helper T cells lines. T cell lines established with IL3 produced by the WEHI 3 cell line eventually do not require addition of the WEHI 3 supernatant for continuous growth. These cells will produce small amounts of IL2 following appropriate antigenic stimulation (134, 152, 153, 154).

IL3 appears to be identical to "P (precursor) cell growth factor" and "burst promoting activity for erythroid cells". IL3 is also active in inducing growth and differentiation of mast cells and other granular cells (155). IL3 is also identical to one form of CSF called CSF-multi (J. Watson, personal communication). IL3's primary action seems to be in growing multipotential precursor cells from bone marrow.

IL3 is a glycoprotein of molecular weight 41,000. It is active when purified to apparent homogeneity at 10^{-11} M. This is comparable to the activity of interferon or IL2 (155).

1.6.3 TERMINAL DIFFERENTIATION FACTORS

IL2 alone may not be sufficient for the generation of CL from unprimed cells (57,85,91,122,175,254,256,257,342,356). The factors acting in conjunction with, but not replaced by, IL2 have been designated "terminal differentiation factors" (342). Most of these factors have been identified by characterizing a cell free supernatant which is sufficient for continuous proliferation of CL clones but not sufficient for CL generation. Culture with unprimed spleen cells or thymocytes is usually used as the assay system.

Since antibodies which block immune interferon activity prevent CL generation (175), one of the terminal differentiation factors is probably interferon. Interferon-free IL2 preparations are much less effective in the generation of CL from CL precursors (H.S.Teh, personal communication). Whether the other terminal differentiation factors

identified are also immune interferon is much less certain. Several of the factors have size and other characteristics appropriate for immune interferon (85,166,175).

In some systems, IL2 cannot replace the need for exogenous IL1 or an IL1-like factor in the generation of CL (65,91). One of the terminal differentiation factors has the size and functional characteristics of IL1 (122). Interferon and IL1 may represent two of the "terminal differentiation" factors required for CL generation.

1.7 IL2 IN OTHER IMMUNE RESPONSES

1.7.1 MATURATIONAL EFFECTS OF IL2

Thymocytes are not normally responsive to alloantigens in the absence of exogenous helper T cells (243). IL2 can replace the need for exogenous helper T cells in *in vitro* responses to mitogen (205,234), to alloantigen (283,338,339) or to syngeneic tumor cells (206). Immature thymocytes (PNA^+ $Ly\ 1^+$ 2^+ 3^+) can be driven to mature CL by the addition of IL2-containing medium and antigen (55, 56, 57, 338, 339). A possible requirement for other factors cannot be ruled out. Highly purified IL2 (56) or supernatants lacking terminal differentiation factors (85,122,254) are less effective in generating CL than are crude IL2-containing preparations.

The maturation of T cells in neonatally thymectomized mice can be driven by IL2 *in vitro* or *in vivo* (299). This induction of T cell reactivity is antigen independent (299).

Nude mice, in addition to lacking hair, are immunodeficient. They seem to lack some of the functions of the thymus. Nude mice have, therefore, been used as a model for some types of human immunodeficiency diseases. One of the defects in cells from young nude mice is the inability to produce IL2. Cells from young nude mice retain the ability to produce IL1 and to respond to IL2 (291). Injection of IL2 and allogeneic cells into nude mice (which do not seem to possess an IL2 inhibitor in their serum, 338,339) results in the generation of CL specific to allogeneic H2 antigens (339). *In vivo*, exogenous IL2 causes nude mouse cells to mature to the normal Thy 1^+ Ly 2^+ 3^+ phenotype of effector CL (339). IL2, *in vitro*, allows the generation and continuous proliferation of CL from spleen cells from nude mice (104,291). The maturation of nude mouse spleen cells by IL2 *in vitro* may be following an abnormal pathway as both H2 restricted and H2

non-restricted CL can be generated *in vitro* (111,339) rather than just the expected H2 restricted CL (365).

1.7.2 GENERATION OF NATURAL KILLER CELLS

To determine whether a cultured cells belongs to the NK lineage or to the CL lineage is at present difficult, if not impossible. CL can only kill cells expressing major histocompatibility antigens. Additionally, CL normally recognize foreign antigens in conjunction with "self"-major histocompatibility antigens. CL react to specific antigenic structures. In contrast, NK cells are not "restricted" by any of the above requirements. NK cells seem to kill targets on the basis of changes in carbohydrate, lipid, or sialic acid content rather than on the basis of specific antigenic structures. Despite these differences, it is difficult to separate activities meditated by NK cells from those mediated by CL.

Murine NK cells can be derived from a Thy 1$^-$ population. Following culture, NK cells express significant quantities of Thy 1 on their surface along with other antigens expected on mature T cells (175). This suggests that the NK cell precursor may be an immature cell of the T lineage. Mature T cell markers are also seen on human NK lines (229,286).

NK cells respond to a T cell growth factor, IL2, with continuous proliferation and increased activity (65,66,175,302,333). Whether the increased NK activity is directly related to IL2 or to IL2 induction of interferon production (85,133,166) is not yet clear. The activity of NK cells can be significantly inhibited by an anti-IL2 antibody, also suggesting a direct role for IL2 in the maintenance and propagation of NK cells (70). NK cells can be cloned in the presence of IL2-containing supernatants (302,333); once again suggesting a role for IL2 in NK cell generation. Unlike CL, NK cells respond to IL2 equally well with, or without, exogenous antigen. Whether this indicates previous antigen exposure or a cell lineage different from "classical" T cells is not clear.

NK cell clones lyse many different cell lines. This suggests that NK cells can recognize a common structure or structural change on all NK-sensitive cell lines (66). Alternatively, NK cells may carry many different antigen receptors on their surface (66). NK cells may simply be a subset of CL acting against a common structure expressed by all

NK-sensitive cells.

1.8 IL2 IN IMMUNOLOGIC DISEASES

1.8.1 IL2 AND AGING

Cells from old mice (43,318) or cells from old rats (112) exhibit decreased IL2 production and decreased responses to IL2. Abnormalities of Ia recognition may be the cause of abnormalities in lymphokine regulation (43,112). Decreased numbers of IL2 receptors have also been implicated (43,112,269). This change in IL2 production and response may relate to the decrease in immune responses observed in cells from older animals along with the increases in autoimmune and malignant diseases seen in these animals.

1.8.2 AUTOIMMUNE DISEASE

Strains of mice expressing high frequency of autoimmune diseases also express decreased production of IL2 and decreased response to IL2 (5). This defect is particularily marked in old mice and occurs about the same time as the onset of autoimmune disease (5). The observed decrease in the activity of the immunostimulatory factor, IL2, seems paradoxical when the plethora of autoimmune responses and the generalized B cell hyper-activity seen in these animals is considered (5). Perhaps autoimmune diseases result from decreased effectiveness of IL2 on IL2-sensitive suppressor T cells. Decreased suppressor cell activity has been demonstrated in at least one model of autoimmune encephalitis (16).

1.8.3 IMMUNODEFICIENCY DISEASE

Cells from patients with a variety of primary immunodeficiency diseases produce decreased amounts of IL2 in response to mitogen stimulation (189). Those cells with decreased proliferative responses to phytohemaglutinin demonstrated decreased production of IL2 (189) and are sensitive to exogenous IL2. These preliminary results suggest that, in some human immunodeficiency states, abnormal IL2 production may play a role. The immunodeficient nude mouse also demonstrates abnormalities in IL2 production

(291). It will be of interest to determine if exogenous IL2 *in vivo* will improve the immune response of some of these patients as it does in the nude mouse (339).

1.8.4 IMMUNOPROLIFERATIVE DISEASE

The Sezary syndrome is the result of an abnormal proliferation of IL2-producing cells (296). Several human cell lines and malignant cell explants produce and respond to IL2 (121). The autostimulatory properties of IL2 may well play a role in these immunoproliferative diseases (121,296). Sensitive assays for IL2 reactivity, such as blocking proliferation by anti-IL2 antibodies or determining the presence of IL2 receptors, suggest that production of IL2 and autostimulation by IL2 may be a common phenomenom (85).

1.9 MODEL OF CL GENERATION

The experimental evidence for each step has been presented in the preceeding sections. Where possible, this model has been developed and documented for the generation of specific CL. Because of the extreme complexity of the *in vivo* control network, this model has primarily been developed from *in vitro* systems. Although the purpose of this model is to obtain a better understanding of host-tumor interactions, the generation of CL directed against syngeneic tumor antigens has proven to be very difficult (58, 267). Therefore, the model presented was derived from the generation of CL directed against allogeneic antigens. It is hoped that the alloantigen model system will be similar to that which occurs in the host-tumor environment.

A stimulating cell (S), exhibiting antigenic determinants recognizable by the host immune system, contacts the immune system (Figure 1). This contact activates a T cell (Ta) which communicates with macrophages probably through the soluble factor, CSF-GM (Step I). Ia recognition and binding may result in activation or it may play a passive role in activation by keeping the Ta cell and the macrophage in close proximity. The macrophage, activated by antigen and CSF-GM, then secretes IL1 (Step 2). Phagocytosis of antigens and presentation of antigens by macrophages may be important for antigen clearance but it is not necessary for the induction of CL by allogeneic antigens.

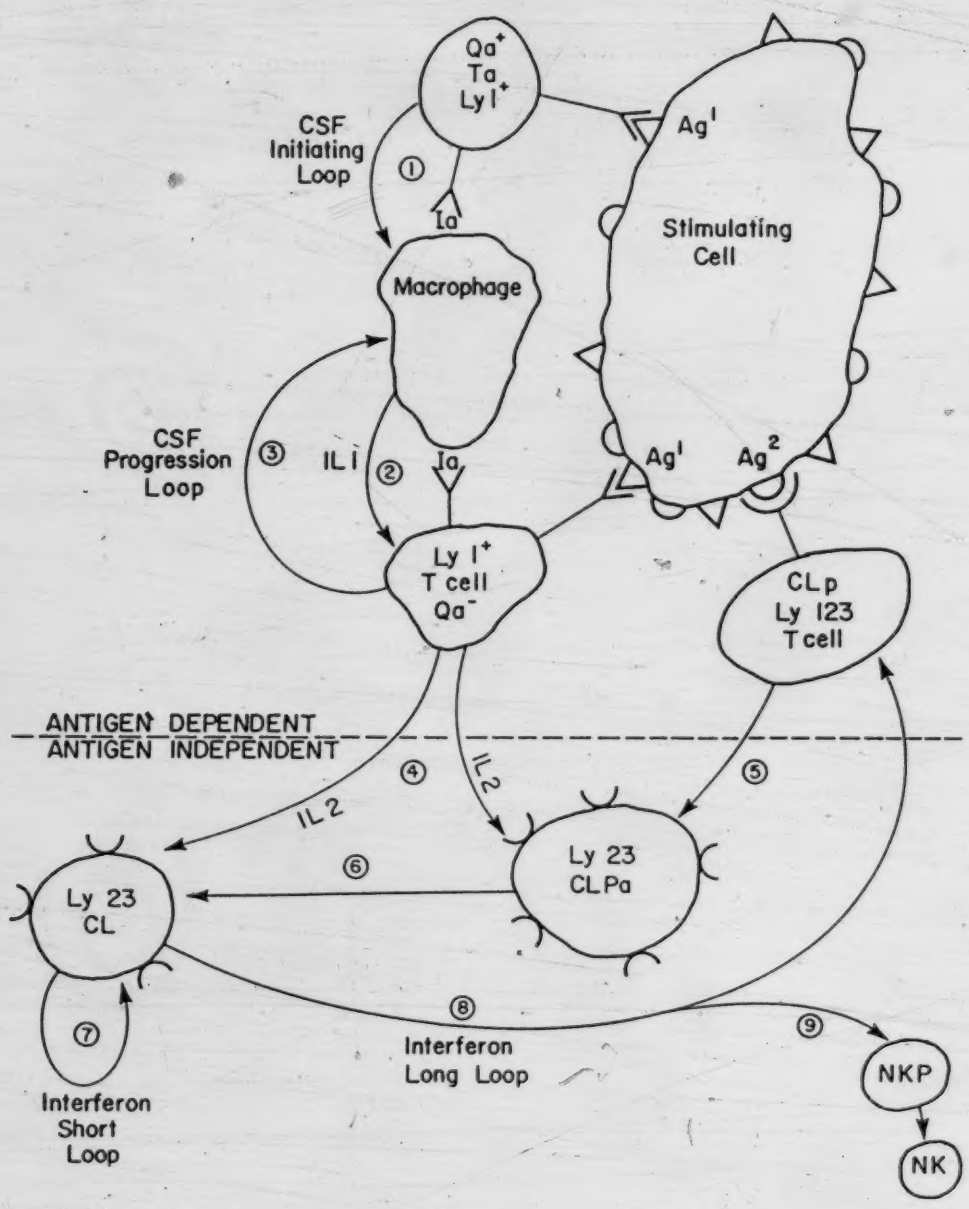

Figure 1: In vitro generation of CL

Positive regulation

IL 1 then activates a Ly I+ T cell to secrete IL2 (Step 4). Antigen is required. The IL2 producing cell may also produce CSF-GM leading to continued IL 1 and IL2 production (Step 3).

Antigen on the stimulating cell results in the expression of IL2 receptors on the surface of CL precursors (CLP) (Step 5). Macrophages, Ia, or soluble factors are not required. The antigen's conformational characteristics can be different from those leading to the production of IL2. Following the induction of IL2 receptors, the generation and production of CL is antigen-independent.

IL2 (and perhaps other differentiation factors) causes the maturation of activated CLP (CLPa) into effector CL (Step 6). Effector CL can proliferate in IL2-containing medium in the absence of added antigen. These cells retain antigen specificity.

The CL also produce interferon. This interferon acts through a short loop (Step 7) to maintain IL2 receptors on the CL. Interferon may also act on CL precursors to generate IL2-sensitive CLPa (Step 8). This could explain the high level of non-specific CL or NK-like cells seen in cultures with high concentrations of exogenous IL2. The exogenous IL2 could contain interferon or induce the endogenous production of interferon. Interferon could also directly result in the generation of NK cells (Step 9). These NK cells may play an additional role in tumor clearance.

A comparison of the characteristics of the factors involved in CL generation is included in Table 1 (Table 1).

1.10 NEGATIVE REGULATION OF CL GENERATION

Only the stimulating factors for CL generation have been described. Negative feedback ensures that the immune system returns to a baseline state following induction. Controlling systems must, of necessity, be present to prevent "by-stander reactions" from causing autoimmune disease and to prevent immunoproliferative disease.

Since IL2 is one of the final factors in the activation cascade (CSF-GM-IL 1-IL2-interferon), blocking production of CSF-GM or IL 1 will result in decreased IL2 production. Such a block can be genetic, such as the inability of the nude mouse to produce IL2 in response to IL 1 (291); constitutive, such as suppressor cells in human peripheral blood (224); or inducible, such as suppression by activated T cells (131)

Table I

Comparison of Factors in CL Activation Cascade

	CSF-GM	IL1	IL2	Immune Interferon
Molecular weight				
Mouse	25-35,000	12-18,000	30,000	30,000
Human		12-18,000	15,000	30,000
Rat			15,000	
Protease sensitive	+	+	+	+
Carbohydrate		+	+?	
H-2 nonrestricted	+	+	+	+
Species restriction	?	no	variable	yes
Cell of origin	T helper cell Fibroblasts	macrophage	T helper cell	T cell
Continuous proliferation of CTL	no	no	yes	?
Direct antitumor effect	no	no	no	yes
PH2 sensitive	?	no	no	yes
Isoelectric points				
Mouse		4.5-5.5	3.5-5.5	
Human		6.5-7.5	6.8-7.2	
Ia surface antigens		no	no	no

or suppression by products from activated T cells (170).

1.10.1 SUPPRESSOR CELLS OF IL2 PRODUCTION

Some suppressor cells decrease IL2 production (131,224). Soluble factors replacing these cells were not identified (131,224). However, a another group of researchers has isolated a 10,000 molecular weight suppressor factor from alloantigen-activated cells (170). This suppressor factor decreases IL2 production (170).

Suppressor cells involved in allowing malignant cell growth may have at least one of their actions via inhibition of IL2 production (20,158,308). Spleen cells from tumor-bearing mice (206) and peripheral blood cells from cancer patients (330) contain increased numbers of CL precursors. The CL activity can be unmasked by culture in IL2 suggesting that failure of IL2 production prevents an effective anti-tumor response *in vivo*.

1.10.2 PHARMACOLOGIC SUPPRESSION OF IL2 PRODUCTION

Corticosteriods decrease murine CL generation by decreasing IL2 production. The blockade is indirect. Corticosteroids act through an inhibition of both IL1 production and effect (103,180,181,291,295). Corticosteroids may prevent IL1 production by blocking Ia expression by macrophages (295). Corticosteriod induced suppression of CL generation can be completely abrogated by addition of high concentrations of IL1 or IL2. The IL2 responsive cells, therefore, are not directly affected by corticosteroids (291). Similar results have been found in human systems (224).

Cyclophosphamide, a potent chemotherapeutic and immunosuppressive alkylating agent, blocks CL generation by preventing IL2 production (198,199). CL generation can be recovered *in vitro* (198) or *in vivo* (199) by treatment with IL2-containing supernatants. The site of action of cyclophosphamide on the IL2 cascade is unknown. Cyclophosphamide, at lower concentrations, also inactivates suppressor T cells (129,222,266) thus decreasing production of an IL2 inhibitor (135). The end effect of addition of cyclophosphamide to any system, therefore, reflects the balance of cyclophosphamide's effect on helper T cells and on suppressor T cells.

Histamine, bound to histamine receptors on T cells, seems to play a significant role in modulation of IL2 production. Pyrilamine, a histamine antagonist specific for the H1 receptor, decreases IL2 production without decreasing the ability of cultured cells to respond to exogenous IL2 (305). Conversely, cimetidine, a histamine antagonist specific for the H2 receptor, increases IL2 production (224). Histamines through the two receptor types can, therefore, have opposing effects on IL2 production and thus on CL generation.

Cyclosporin A is an immunosuppressive agent which facilitates transplantation of various organs in animals and humans (240). In human model systems, cyclosporin A has been reported to significantly decrease IL2 production (141). In murine model systems, cyclosporin A prevents IL2 production from unprimed cells by preventing expression of IL1 receptors thus preventing response to IL1 (8,239). Additionally, cyclosporin A prevents IL1 release from stimulated macrophage cell lines and probably from macrophages following physiologic stimulation thus, indirectly, preventing IL2 production (8). Depending on the nature of the stimulus and the maturity of the responding cell, cyclosporin A can prevent both IL2 production and IL2 receptor induction (71). Cyclosporin A inhibits primary responses more effectively than secondary or primed responses (71). The failure of cyclosporin A to block primed responses may limit its possible therapeutic usefulness. Perhaps the facilitation of transplantation observed following cyclosporin A therapy is through the prevention of IL2 effects and thus prevention of CL generation.

Prostaglandins, potent local regulators of immune responsiveness, decrease IL2 production from murine (13,293,243) and human cells (53,255). Removal of prostaglandin E-producing macrophages or inhibition of prostaglandin E-producing macrophages by the prostaglandin synthetase inhibitor, indomethacin, increases IL2 production (255). Prostaglandins can decrease the effect of IL2 on antigen-activated cells (13,293,316). Macrophages, by producing both IL1 and prostaglandin E, are capable of producing both inhibitory and stimulatory effects on the interleukin cascade (293).

CSF-GM, which activates macrophages to release IL1, also increases release of prostaglandins from macrophages (216). CSF-GM, therefore, demonstrates both positive and negative modulatory effects.

1.10.3 INHIBITION OF IL2 EFFECT

IL2 concentrations in supernatants of stimulated T cells peak between 24 and 48 hours (110,283). Thereafter, IL2 responsive T cells develop IL2 receptors and by adsorption and degradation decrease the amount of IL2 in the culture supernatants (85,175,234,259). At least one form of suppressor T cell seems to mediate its effect solely through adsorption of IL2 (237).

Mouse serum contains a soluble IL2 inhibitor. This inhibitor is present in high concentrations in some strains of mice (135,339). The factor has not been shown to block IL2 directly or to be overcome by addition of purified IL2 suggesting that it may react with the IL2 responsive cell rather than with IL2 directly (135). The inhibitor has an apparent molecular weight of 50,000 and is produced by LY 2^+3^+ T cells. Its action is antigen non-specific and H2 and species non-restricted. Production of the IL2 inhibitor is increased by strong antigenic stimuli (135). This inhibitor may play a significant role in maintaining the short range of activity of IL2 and in down regulating active responses (135).

Both specific and non-specific suppressor cells can prevent responses to IL2 (305). This suppressor activity seems to be mediated through inhibition of the antigen-dependent expression of IL2 receptors (305).

1.11 POSSIBLE USE OF IL2 IN GENERATION OF CL FOR CANCER THERAPY

Treatment of human cancer by immunologic intervention holds great promise. Unfortunately, other than anecdotal cases, clinical immunotherapy regimens have failed to fulfill this promise (58,267,351). These failures result from the inability to modulate in a controlled manner the complex cellular and subcellar interactions present in the cancer patient. It may be difficult to specifically alter any one element of this complex system without causing a large scale rebalancing (138). Tumor-bearing patients have, *in situ*, an immunologic system balanced in favor of tumor growth which is difficult to modulate (58,267).

The difficulty in modulating the *in vivo* system directly has made the possibility of *in vitro* manipulation of immune cells, with subsequent infusion of these programed cells, attractive (86,207,267). The requirements for successful adoptive immunotherapy would

be:

1). ability to grow active cells from tumor patients *in vitro* (267),

2). ability of these active cells to home to tumor targets *in vivo* (351), and

3). ability of these cells to change the local balance of the immune system towards tumor clearance in patients with established tumors (267).

Although the definitive cell, if any, involved in *in vivo* cancer control has not been identified, CL, or cells generated concomitantly with CL, are attractive candidates for possible immunotherapy trials (86,267). Therefore, generation of CL *in vitro* with the goal of reinfusion of these cells is a possible technique for bypassing some of the complex control mechanisms in place in the tumor-bearing patient (86,267).

Culture of cells from tumor-bearing animals with IL2 or IL2-containing supernatants may allow the generation of tumor-specific CL. These CL, when injected into tumor-bearing mice, may be an effective immunotherapy technique. The remainder of this thesis explores this mode of therapy.

1.11.1 CONSTRAINTS ON MODEL SYSTEMS

A valid animal model for developing immunotherapeutic techniques should develop and assess effector cells in animals with established tumors. It is particularily important for assays of both the induction and the effectiveness of therapeutic populations that tumors be present long enough to establish the suppressor mechanisms which normally prevent the induction of effective host responses in tumor-bearing animals (20,32,138,158,309).

For example, a Winn (tumor neutralization) assay involving injection of admixed effector cells and tumor into normal mice assesses a different cell function from that effective in clearing established tumors (105,260,355). In most systems, the Winn assay correlates with *in vitro* cytotoxicity assays. Cells active in Winn assays often are totally ineffective in treatment of established tumors (37,105,260). The Winn assay is, therefore, not a good assay for possible immunotherapy techniques. Similarily, cells generated from immune mice or from mice with spontaneously regressing tumors cannot be taken as general models for generation of CL from tumor-bearing patients (14,187,351). Therefore, models involving generation of effector cells from

tumor-bearing animals and use of these effector cells in tumor-bearing hosts will be considered as the most relevant.

1.11.2 IDENTIFICATION OF THE CELL ACTIVE IN IMMUNOTHERAPY

Cell mediated immune responses can play a role in controlling malignant cell growth (19,208,267). The relevance of anti-tumor responses in immunized animals to the spontaneous human tumor situation is questionable (14,351). The presence of anti-tumor cell mediated immune responses is well established, but their magnitude in the tumor-bearing host is uncertain (14). Although cell mediated immunity can play a role in tumor clearance, identification of the cell type(s) involved has been difficult (129,191,207). Candidates include NK cells (262,352), DTH mediating cells (192), CL^{+} (207,328), helper T cells (129), and activated macrophages (329). It is very likely that each of these cell types along with non-specific accessory cells play a role in tumor clearance depending on tumor cell histology and stage of tumor growth.

1.11.2.1 NK CELLS AS CANDIDATES FOR TUMOR IMMUNOTHERAPY

Beige mice, which are deficient in NK activity, are more susceptible to some syngeneic tumors than are their heterozygous litter mates (157,164). The Chediak-Higashi syndrome in humans is manifested by an increased incidence of pyogenic infections and spontaneous tumors. These patients demonstrate significantly decreased NK cell activity with apparently normal CL activity (262,264). Since NK cells may play a role in tumor surveillance (263,264), immunotherapy with NK cells may be effective, particularly in patients with very small residual tumors.

1.11.2.2 HELPER CELLS AS CANDIDATES FOR TUMOR IMMUNOTHERAPY

For adoptive transfer of immunity from tumor-immune animals, the effective cell can be shown to be a $Ly\ 1^{+}$ cell of helper or DTH phenotype (129). The rejection of allogeneic skin grafts (191) and the rejection of allogeneic tumors (192) is also mediated by $Ly\ 1^{+}$ T cells. $Ly\ 2^{+}3^{+}$ CL are both unnecessary and incapable of mediating rejection. Since helper cells may play a role in these models, they may also play a role in immunotherapy of syngeneic tumors.

1.11.2.3 CYTOTOXIC LYMPHOCYTES AS CANDIDATES FOR TUMOR IMMUNOTHERAPY

Although CL can be grown directly from tumors or the blood of tumor patients (190,362), the role of CL in immune surveillance is not clear (351). Nude mice, which are incapable of mounting CL responses, do not seem to have a significantly increased incidence of spontaneous tumors (263,352). The presence of increased NK cell activity in nude mice along with their generally short life span may explain the lack of increase in spontaneous tumors (263). Since nude mice do not reject transplanted syngeneic, allogeneic or even xenogeneic tumor grafts, tumor clearance mechanisms in nude mice are clearly abnormal.

When tumor cells from tumors which normally grow and then spontaneously regress *in vivo* are cultured with normal spleen cells, tumor cell lines which kill syngeneic animals develop (329). The new tumor lines are sensitive to NK activity but resistant to killing by CL or macrophages. The *in vivo* growth of these tumors suggests that, for these tumors, NK cells play little or no role in protection from tumor growth. CL and macrophages play the controlling role (329). In this system, all of the cell lines are resistant to both macrophage and CL killing, suggesting that either CL or macrophages are capable of preventing tumor growth.

Virus-induced tumors in mice often grow, for a time, and then spontaneously regress (187). The presence of tumor-specific CL in these mice suggests that CL play a role in the control of these viral induced tumors (187,195). The relevance of this model of spontaneously regressing viral tumors to human tumor systems is questionable (187,351).

Tumor patients (190,330,362,364), tumor-bearing mice (64,206,236,361), and tumor-bearing guinea pigs (17,18) have cells which can kill autologous or syngeneic tumor cells. Separation from suppressor cells followed by culture (17,18,64,362) or culture with IL2 (190,206,236,330,361,364) is necessary to unmask the anti-tumor activity. These populations can prevent tumor growth *in vivo* in Winn assays (17,18,207,236) or in the clearance of established tumors in immunosuppressed mice (236). It is otherwise difficult to assay CL activity from tumor-bearing animals. CL activity could only be demonstrated by incubating very high ratios of effector cells with target cells (194); by

incubating effector cells with target cells for long periods (308); or by cytostasis of tumor growth (15).

We have used the induction of CL activity as an indication of general anti-tumor activity. As will be demonstrated later, the CL population generated contains helper cells capable of recruiting host responses, CL capable of killing tumor cells, and NK-like cells capable of killing tumor cells.

1.11.3 HOMING OF EFFECTOR CELLS TO TUMOR

Although it is well established that cells from tumor-immune hosts can home to tumor cells, the ability of cultured cells reinfused into mice to home to tumor cells is much less certain (37,60,105,190,207,260). CL generated *in vitro* generally survive for very short periods of time *in vivo* (105,190,207,260). They home to different host organs than do fresh cells. A large proportion of cultured cells are trapped in the lung (37,60,190). DTH mediators generated *in vitro* (159), helper T cells generated *in vitro* (314), and suppressor T cells generated *in vitro* (16) seem to home more effectively than do CL generated *in vitro*. Thus, these cell types mediate more *in vivo* activity than do CL generated *in vitro*. The inability of CL generated *in vitro* to home to tumors needs to be overcome before immunotherapy with CL generated *in vitro* can be added to the armamentarium of the oncologist.

1.12 HISTORY OF HUMAN ADOPTIVE IMMUNOTHERAPY

Despite the difficulties and limited success with animal models, many clinical trials of transfer of immune cells have been performed (58,267). Initially, lymphocytes from melanoma patients, who had had spontaneous regression of tumors, were injected into patients who had active metastatic melanoma (267). Several of the treated patients exhibited remarkable regression of tumor nodules. Because melanoma tumors occasionally regress spontaneously and since these trials were not adequately controlled, it is difficult to attribute these responses to the therapy.

Infusion of large numbers of normal lymphoid cells into cancer patients (267) generated little or no anti-tumor response. Evaluation of this form of therapy has been complicated by the use of concomitant chemotherapy (307,357), the use of

spontaneously regressing tumors (307) and generally from the lack of use of proper controls. Several workers have attempted trials with cells immunized to tumor antigens either *in vivo* (7,149,217) or *in vitro* (217). In the initial trials, therapy with autologous human lymphocytes cultured *in vitro* failed to mediate *in vitro* or *in vivo* cytotoxicity (217). No adverse side effects were noted. In an uncontrolled follow-up study, 154 patients were matched by tumor types (218). Untreated tumor from each partner was then transplanted into the other. After this immunization, peripheral blood or lymphatic duct lymphocytes were isolated. When these alloreactive lymphocytes were injected into the original tumor donor, 20% of the patients were thought to have subjective partial remissions. 3 out of 118 patients were tumor-free at the time of the report (4 1 /2, 2, and 1 year post-therapy). The responders either had tumors which occasionally spontaneously regressed (malignant melanoma), had unproven metastases or were receiving chemotherapy. Side effects were quite spectacular. Two patients developed tumors at the site of the tumor allograft despite previous immunization to the donor's histocompatibility antigens. Simple excision prevented further tumor spread. One patient died of GVH disease. His lymphocytes had been replaced completely by lymphocytes exhibiting the female phenotype of the lymphocytes he had received in therapy. Dismal results were obtained by other workers using similar techniques (7). No clinical responses were noted and one patient died as a direct result of the therapy. Another study of cross-immunized patients demonstrated clinical responses in 8 of 38 patients and objective results in 6 of 38 patients (149,150). No significant side effects were noted.

A more recent human study combined immunotherapy with non-specific adjuvant cells activated by culture (267). These melanoma patients had all failed to respond to conventional therapy. Despite all patients having recurrent disease with multiple metastases, 6 of 11 patients were tumor free 30 months after treatment. *In vitro* assays of immunocompetence were significantly improved in responding patients. Because the trial was uncontrolled, it is once again difficult to assess whether the responses were a result of the immunotherapy or due to the natural course of the disease.

Human lymphocytes cultured with exogenous IL2 express *in vitro* activity against autologous malignant melanoma (190). Lymphocytes from patients with sarcoma were cultured in IL2, then radiolabeled with ^{111}In-oxine. They were then injected back into the

donor. No adverse side effects or clinical responses were noted following this procedure. Most of the cells were trapped in the lung and did not travel in the same pattern as normal lymphocytes (190). Homing to tumor cells could not be demonstrated. Because the *in vitro* cytolytic activity of the infused lymphocytes was not determined (190), it is difficult to determine if this preliminary study has any relevance to possible therapy with *in vitro* generated CL-containing populations.

These studies point out the difficulties and dangers of using allogeneic lymphocytes. Autologous sensitized lymphocytes may be effective if large numbers of sufficiently active cells can be obtained. These cells, even if active *in vitro*, may not be effective *in vivo* because they are unable to home to the tumor cells.

1.13 THESIS

From a review of data on the generation of CL and data on the effectiveness of CL in tumor eradication, the following working hypothesis was developed: progressing tumors fail to elicit an immune response capable of eradicating the tumor not because the tumor cells lack antigenic determinants but rather because the response to the tumor is suppressed. This suppression can act on the IL2 cascade. Culturing cells from tumor-bearing animals with IL2 may elicit effective immune responses. Cells mediating these responses should then be capable of tumor clearance *in vivo*. The testing and partial verification of this hypothesis constitute the remainder of this thesis.

Chapter 2 describes the materials and methods used to develop the immunotherapy model.

Chapter 3 describes the techniques developed for the generation of cytotoxic activity from tumor-bearing mice. This activity is compared to that generated from cells from normal mice cultured with syngeneic and allogeneic tumors. The origin of the cytotoxic activity developed *in vitro* is explored. Tumor-specific cell lines were developed to allow characterization of the effector cells. Preliminary applications to human cancer patients are described.

Chapter 4 describes the *in vivo* activity of these anti-tumor cells. The origin of the transferred activity along with the participation of host cells in anti-tumor activity is discussed.

Chapter 5 describes techniques for increasing the efficacy of the anti-tumor activity generated *in vitro* in therapy of animal models of human tumors. The advantages of combining CL therapy with surgery and repeated injections of CL to eliminate residual tumor are discussed.

Chapter 6 summarizes the data presented and suggests directions for further animal experiments prior to consideration of this technique for therapy of human tumors.

2. MATERIALS AND METHODS

2.1 TISSUE CULTURE MEDIUM AND INCUBATION CONDITIONS

RPMI 1640 medium was obtained from either Flow Laboratories, Rockville, Md., or from GIBCO Laboratories, Grand Island, N.Y., and was supplemented with 20 mM sodium bicarbonate, 0.34 mM pyruvate and 0.02 M HEPES pH 7.3 (Sigma, St Louis, Mo.). Antibiotics, either 40 micrograms / ml gentamycin sulfate (Garamycin, Ayerst Laboratories, Montreal, Quebec), or 50 micrograms / ml Penicillin G potassium and 74 milligrams / litre streptomycin sulfate (GIBCO), were added to the medium. Mercaptoethanol, 5×10^{-5}, and 10% v / v fetal bovine serum (Flow or Gibco) were added to all culture medium. Fetal bovine serum was inactivated at 56°C for 30 min. Medium was sterilized by filtration through 0.45 micron filters (Millipore, Mississauga, Ont).

Cell cultures were incubated in a humidified atmosphere of 5% carbon dioxide in air at 37°C.

2.2 PREPARATION OF SPLEEN CELLS AND IL2 PRODUCTON

Mice were killed by cervical dislocation and their spleens were removed into tissue culture medium. To obtain a suspension of spleen cells, the spleens were minced with scissors and forced through a stainless steel grid. Clumps were allowed to settle out for 5-10 min. The cells were then washed twice in tissue culture medium by pelleting at 750 x g for 7 minutes, decanting the supernatant, and resuspending the cells in fresh medium. Cell viability was determined by staining with 0.14% eosin Y in saline.

For IL2 production, the spleen cells were cultured at a density of 12×10^6 cells / ml in medium without fetal bovine serum. They were stimulated with 1.5-3 micrograms / ml Concanavalin A (Con A) (Calbiochem., La Jolla, Ca.) for 18 to 24 hr. Cultures, 200-250 ml, were set up in Blake tissue culture bottles (surface area 250 cm^2). After incubation for 24 hours, the cells were pelleted at 750 x g for 7 minutes and the supernatant was decanted. This supernatant is referred to as crude IL2.

2.3 PURIFICATION OF MOUSE IL2

2.3.1 AMMONIUM SULFATE PRECIPITATION

Ammonium sulfate precipitation was performed at 80-90% saturation. Solid ammonium sulfate was added slowly to the culture supernatants with gentle stirring. After 15 hours at 4°C, the precipitate was collected by centrifugation for 15 min at 10,000 x g or for 1 hr at 4,000 x g. The precipitate was redissolved in a small volume of distilled water.

The specific activity in units / mg protein prior to ammonium sulfate precipitation was 180 and following precipitation and redisolving in 1 / 100 of the original volume, 170. The yield was 30 %.

2.3.2 SEPHADEX G-100 CHROMATOGRAPHY

A 2.6 x 100 cm column (500 ml) of Sephadex G-100 (Pharmacia, Dorval, Quebec) was equilibrated in buffer A (0.05 M NaCl, 0.01 M HEPES - pH 7.3). The void and total volumes of the column were determined using Blue Dextran 2000 and ^{3}H-thymidine, respectively. Samples applied to the column ranged in volume from 10-50 ml. Five ml fractions were assayed on thymocytes as described below. Fractions of peak activity were pooled and designated as fraction 3 IL2. Chromatographic runs were done at 4°C.

The specific activity of fraction 3 IL2 was 270 units / mg. There yield was 100%.

2.3.3 DEAE-SEPHACEL CHROMATOGRAPHY

DEAE-Sephacel (Pharmacia) was equilibrated in buffer A containing 0.2 mM EDTA and transferred to a 0.9 x 15 cm column (8 ml). Fraction 3 IL2 was applied to the column and the column was washed extensively with starting buffer. Activity was eluted with a linear salt gradient of 0.05 M to 0.35 M NaCl. Fractions of peak activity were pooled and are referred to as fraction 4 IL2. Chromatographic runs were done at 4°C. Unless otherwise stated, all experiments were performed with fraction 4 IL2.

The specific activity of fraction 4 IL2 was 1,500 units / mg. The yield was 100%.

2.3.4 SDS TREATMENT OF IL2

Sodium dodecylsulfate (SDS)-treated and SDS-column purified IL2 was kindly provided by B. Caplan. IL2 was denatured by heating at 90°C for 2 min in 1% SDS and 10 mM dithiothreitol (DTT) and then passed over a calibrated Sephacryl S-300 column equilibrated in 0.1% SDS. SDS was precipitated from the collected fractions by cooling (40).

Specific activity was 20,000 units/mg which represents a purity of about 2 %. The yield was approximately 50%.

2.4 PREPARATION AND PURIFICATION OF HUMAN IL2

Peripheral white blood cells from 2 donors were collected by venipuncture and separated on Ficoll-hypaque (Pharmacia). Lymphocytes, 2 x 10^6/ml from each donor, were combined and cultured for 24 hours with 24 ugm/ml Concanavalin A (Con A) and 2 x 10^6/ml irradiated Daudi or 6140 cells. Medium was removed and fresh Con A containing medium was added for an additional 24 hours. Both preparations were pooled and designated crude human IL2.

The crude IL2 was freeze dried, dissolved in distilled water, and passed over a G10 column (Pharmacia) to remove salts and most of the Con A.

Sephadex G-100 chromatography was the same as for mouse IL2 purification except the column was of much smaller size (1.5 x 50cm, 100ml). The void and total volumes were determined with tritiated E coli DNA and ^{14}C thymidine, respectively.

2.5 MONOCLONAL ANTIBODIES

Monoclonal anti-Thy 1.1 antibodies were obtained from NEN, Lachine, Quebec. Monoclonal anti-Ly 1.1 antibodies were a gift from Dr.I.F.C. Mackenzie.

2.6 MICE

Male DBA/2J mice were obtained from Jackson Laboratories (Bar Harbour, Maine). Male CBA/J and CBA/CaJ mice were bred locally. All mice were housed in the University of Alberta Health Sciences Animal Center.

2.7 CELL LINES

P815, a DBA/2J mastocytoma (H2-d); L1210, a DBA/2J lymphocytic leukemia (H2-d); CaD2, a DBA/2J mammary adenocarcinoma (H2-d); S194, a Balb/c myeloma (H2-d); MOPC 21/P3 (P3), a Balb/c myeloma (H2-d); EL4, a C57/Bl6 lymphoma (H2-b); RI, a CBA/J lymphoma (H2-k); BW5147, an AKR lymphoma (H2-k); and 6140 and Daudi, human lymphoblastoid lines, were maintained in tissue culture. P815 was passaged through DBA/2J mice once a month. "Metastatic" P815 cells were generated by repeated subcutaneous injection of P815 cells. The P815 cells which subsequently grew from cultured spleen cells were designated "metastatic" P815.

2.7.1 HUMAN OVARIAN CARCINOMA LINE, A.F.

Dispersed ovarian cystadenocarcinoma cells from both the solid tumor and ascitic fluid from patient A.F. were cultured in 10% v/v crude human peripheral lymphocyte conditioned medium with added alpha-methylmannoside and 3 units/ml murine IL2. Every 3-4 days viable adherent cells were selected and recultured at 1×10^4 cells/ml. After a period of one month in culture, cells began to grow autonomously. Cell division times were 36-48 hours. Conditioned medium was not required for further growth. When examined histologically (L. Honore Dept.of Pathology, University of Alberta), these cells were not fibroblasts and morphologically were identical to the original tumor cells present in the patient's ascitic fluid. This tumor line was designated A.F.

2.8 TUMOR-BEARING MICE

2.8.1 P815

DBA/2J mice were injected subcutaneously (s.c.) with 1×10^3 to 1×10^4 P815 cells. Tumors were palpable by day eight and the mice all died by day 30. As early as 7 days after s.c. injection, metastatic P815 could be cultured from spleen or liver. The minimum lethal tumor dose for P815 was 10^2 cells i.p. and 5×10^2 cells s.c. (data not shown).

2.8.2 OTHER TUMORS

RI and L1210 cells were injected s.c. at 1×10^3 cells/mouse. CaD2 was injected at 1×10^4 cells/mouse. Mice did not survive these doses of tumor without anti-tumor therapy.

2.9 IL2 ASSAY

The standard assay for IL2 was its ability to cooperate with Con A in stimulating the proliferation of CBA mouse thymocytes cultured at low cell density. It correlates directly with other assays for IL2 activity (40). The concentration of IL2 required to obtain 1/3 of the maximal stimulation in the thymocyte proliferation assay has been defined as 1 unit/ml. CBA mouse thymocytes, 5×10^5 were cultured in 0.2 ml of medium for 72 hours. Con A, 3 ug/ml, was present throughout the assay. ^{3}H-thymidine was added for the last four hours prior to harvesting. Cells were harvested onto glass fibre filters and counted in a Beckman scintillation counter.

2.10 GENERATION OF CL

Optimal concentrations of stimulating cells, responding cells, and IL2 for generation of CL active against syngeneic and allogeneic tumors were similar. Optimal conditions were 1.25×10^6 responding cells/ml cultured with 6×10^4 gamma-irradiated (2500 rad) stimulating cells in RPMI 1640 medium containing 10% fetal bovine serum and 5×10^{-5}M 2-mercaptoethanol. IL2 was optimal at 6 units/ml. Quadruplicate 0.2 ml cultures were set up in 96 well (round bottom) Linbro trays. Each experiment was repeated, unless otherwise stated, between 3 and 5 times, and the results shown are representative.

For experiments characterizing CL, larger numbers of CL were generated in 10 ml cultures in Costar cluster dishes. Microcultures and large scale cultures gave similar values for CL generation.

For large scale CL generation, 1.25×10^6 spleen cells/ml were cultured with 6.25×10^4 gamma-irradiated (2,500 rad) tumor cells/ml. The cells were incubated for five days in 80 ml of medium in 250 ml Corning T flasks. IL2 was added to the cultures in optimal amounts (6 units/ml as determined by thymocyte proliferation assays). Similar

activity was obtained by this technique and the microculture system described above (data not shown). In large scale cultures, CL generation was not as consistant as in microcultures. Only 80% of cultures with P815 cells, L1210 cells, and RI cells resulted in significant CL induction. Only 50% of the cultures with CaD2 cells resulted in significant CL induction. The reason for the discrepancy between the microculture and the large scale experiments is not known but may have been related to the large number of tumor-bearing mice assayed.

2.11 GENERATION OF CL LINES

Cells from cultures of spleen cells with and without added P815 cells were split twice weekly to $2x10^4$ cells/ml. Since the cells were adherent, they were removed from the culture dishes by a one-half hour treatment with 20 mM EDTA in Ca^{++} and Mg^{++} free phosphate buffered saline (pH 7.3). IL2 was added to all cultures at 10 units/ml.

2.12 ASSAY OF CL ACTIVITY

After five days, cells were serially diluted into 96 well Linbro trays. Target cells were added at $1\text{-}5x10^4$ cells/well. Normal DBA cells and DBA/2J lymphoblasts were labeled by overnight culture with ^{51}Cr. DBA lymphoblasts were generated by culturing $3x10^6$ spleen cells/ml for 48 hours in 3 ug/ml Con A. Tumor cells were labeled for 2 hours prior to use. After 5-6 hour incubation in the CL assay, 0.1 ml of supernatant was removed and assayed for specific ^{51}Cr release, F:

$$F=(observed-spontaneous)/(total-spontaneous)$$

Results were plotted according to the relationship suggested by Miller and Dunkley (201):

$$F=1-exp(-NxKAxt)$$

where N is the number of cells cultured to generate the CL, KA is the killing activity, proportional to the number of CL per cell cultured, and t is the time of the assay in hours. KA is similar to the alpha parameter of Miller and Dunkley, differing only in that N in their case refers to the number of cells present in the assay, in ours, to the number of lymphocytes placed into culture at the outset. This parameter is a more useful measure of CL activity than percent ^{51}Cr release, since it accounts for the variables of number of cells cultured, time of assay, and killer:target ratio. In each experiment, KA values for controls

are given, and in some, the relationship of KA to % ^{51}Cr release is also described. Typical values for KA would be: control, $<5x10^{-3}$; significant responses, $20\text{-}200x10^{-3}$. Killer to target cell ratios ranged from 10:1 to 1:1 in each experiment. A 5:1 ratio giving 30% specific ^{51}Cr release in 5 hours corresponds to a KA value of $120x10^{-3}$. Spontaneous release of ^{51}Cr varied from 5 to 15%.

2.13 ANTI THY 1.1 AND ANTI LY 1.1 TREATMENT

Viable cultured cells were selected by centrifugation at 1,500 x g for 1/2 hour on Ficoll-hypaque. These CL were incubated with optimal concentrations of antibodies or normal mouse serum for 1/2 hour at 37° C. This was followed by incubation with rabbit complement for one hour. Viability was determined by eosin exclusion. The cells were then assayed in a standard CL assay.

2.14 NYLON WOOL TREATMENT

Spleen cells cultured for five days with IL2 and P815 cells were incubated in media for 1/2 hour on nylon wool in a 20 ml syringe at 37° C and then eluted with medium. This treatment removes a sufficient number of adherent cells to prevent generation of CL or IL2 (156,283). The resulting population of cells is greater than 95% Thy 1^+ (data not shown) (156,283).

2.15 INTERFERON ASSAY

Murine L cells were cultured in small petri dishes for 5 days. Each plate contained approximitely 5×10^6 L cells at confluence. Culture medium was decanted and the L cells were cultured overnight in 1 ml of medium with IL2 or NIH standard interferon. Each plate was infected with $2.5x10^3$ wild type mengo virus. Plaques were developed 3 hours later. All interferon assays were performed with the assistance of John Bowen, Department of Biochemistry, University of Alberta. Interferon activity of fraction 4 IL2 was kindly confirmed by Dr. K. Paucker, Department of Microbiology, National Institute of Health, Bethesda, Maryland.

2.16 ^{131}I-IUdR LABELING OF TUMOR CELLS

Tumor cells in exponential growth were incubated at 1-2x10^6 cells/ml for 3 hours with 0.5 microcuries of ^{131}I-IUdR/ 10^6 cells. This gave approximately 2x10^5 counts/minute/ 10^6 tumor cells.

2.17 ^{125}I-IUdR LABELING OF CL

Cells from tumor-bearing mice cultured with IL2 and P815 cells for four days were incubated with 1 microcurie of ^{125}I-IUdR/ml for an additional 18 hours. The cells were then washed and injected as described.

2.18 MONITORING OF TUMOR CELL DEATH

A measure of *in vivo* tumor cell killing was obtained by determining the retained radioactivity from injected ^{131}I-IUdR labeled tumor cells by whole-body gamma counting (41). One million radiolabeled tumor cells (3-10x10^5 cpm of ^{131}I) were injected i.p. into each mouse. Each mouse was counted daily in a large well Beckman gamma counter. Each group contained five mice, identified by ear cropping. The retention of ^{131}I was calculated for each mouse, corrected for ^{131}I decay, averaged for the group and then expressed as a percentage of the initial counts. To decrease thyroid uptake of released ^{131}I, 0.1% NaI, final concentration, was added to the drinking water.

Six to seven days after injection of radiolabeled tumor, the ^{131}I remaining in the mice was too low to give an indication of the rate of killing of tumor cells. Because of this, the mice were injected with an additional 1x10^6 ^{131}I-IUdR labeled tumor cells. This second injection of tumor did not affect host survival times (data not shown) (41). Measurement of radioactive iodine clearance enabled an *in vivo* assessment of the response of the host immune system to the tumor and any modification of this result that resulted from immunotherapy.

Cultured spleen cells, usually those surviving from cultures set up with 1x10^7 cells, were injected with a different syringe at least 2 hours after the ^{131}I-IUdR labeled tumor.

2.19 GAMMA AND ULTRAVIOLET (UV) IRRADIATION

Cells and mice were irradiated with 2500 and 600 rads, respectively, from a Cesium source at 100 rad/minute. UV irradiation was for 1/2 hour, 6 cm from a 15 watt germicidal lamp. This dose of UV irradiation was sufficient to kill all of the cells present within 2 hours following irradiation.

2.20 FREEZE-STORED HUMAN TUMOR CELLS

To supply target cells for assessing the activity of human autologous anti-tumor activity, fresh tumor cells were frozen in liquid nitrogen. The freezing mixture was 10% DMSO with 20% fetal bovine serum in RPMI 1640 medium. The tumor cells were thawed and labeled with ^{51}Cr in the same manner as cultured tumor cells. The incorporation of ^{51}Cr was about one-half that of cultured tumor cells. Spontaneous ^{51}Cr release during the cultures was about 30% of the incorporated label.

3. SYNGENEIC ANTI-TUMOR CL

3.1 INTRODUCTION

If tumor-immunotherapy with CL is to be utilized for human therapy, techniques for the generation of large numbers of these cells from cancer patients must be developed. This chapter describes techniques for the development of high levels of anti-tumor cytolytic activity from animal model systems and human patients. The characterization of the cell responsible for this cytolytic activity is also described.

3.2 RESULTS

3.2.1 OPTIMAL CONDITIONS FOR INDUCTION OF SYNGENEIC ANTI-P815 CL

The optimal conditions for generating CL against syngeneic P815 from spleen cells from DBA mice were found to be similar to that for the generation of allogeneic CL (283). Optimal conditions were 1.25×10^6 lymphocytes / ml, 6×10^4 P815 cells / ml and 6 units IL2 / ml (Tables 2-4). Optimal IL2 concentration was twice that for maximal thymocyte proliferation. These conditions were identical for flat bottom micro-culture trays (0.2 ml), small petri dishes (2 ml) or large costar plates (10 ml) (data not shown).

3.2.2 GENERATION OF ANTI-P815 CL BY CULTURE WITH IL2 AND P815 CELLS

Culture of spleen cells from normal or tumor-bearing mice with IL2 and P815 cells generated highly effective CL (Table 5). CL generated from spleen cells of tumor-bearing DBA mice by culture with IL2 and (syngeneic) P815 cells were more active against P815 cells than were CL generated from CBA spleen cells with (allogeneic) P815 cells alone ($KA \times 10^3$ of 1230 and 280 vs 500 and 163, respectively). However, when IL2 was also present, allogeneic spleen cells gave a 10-fold higher response. Significant CL responses against the syngeneic tumor occurred only in the presence of IL2 (Table 5).

The increased response of spleen cells from tumor-bearing mice *in vitro* was demonstrable between 10 and 25 days after s.c. injection of P815 cells. During this time, a higher potential CL activity was present in spleen cells of tumor-bearing mice than in spleen cells of normal mice. This activity was revealed only by incubation with P815

Table 2

Optimal Conditions for Induction of Syngeneic Anti-P815 CL

Responding Cell Concentration

Spleen Cells/ml	KAx10^8		
	Normal DBA	Tumor Bearing DBA	
		Day 14	Day 24
5×10^6	8	225	<5
2.5×10^6	39	492	63
1.25×10^6	108	780	840
6.25×10^5	<5	<5	<5

DBA spleen cells were cultured for 5 days with 1/10 as many irradiated P815 cells. IL2 was present at an optimal concentration as determined for generation of CL against allogeneic cells (3 units/ml) (data not presented) (283) (See Methods). Tumor-bearing mice were injected with 1×10^4 P815 cells s.c. 14 and 24 days before use. Assay was for 6 hours on 3×10^4 ^{51}Cr-labeled P815 cells. Killing activity (KA) is proportional to the number of CL in each culture (see Methods).

Table 3

Optimal Conditions for Induction of Syngeneic Anti-P815 CL

Stimulating Cell Concentration

P815 cells/ml	KAx10^8 Normal DBA	Tumor Bearing DBA 24 Day
0	42	84
5 x 10^5	<5	N.D.
2.5 x 10^5	<5	324
1.25 x 10^5	105	840
6 x 10^4	228	486
3 x 10^4	90	N.D.

DBA spleen cells, 1.25x10^6/ml, were cultured with the specified number of P815 cells for 5 days in microtitre plates. IL2 was present an an optimal concentration as determined for allogeneic CL generation (3 units/ml). Cells were assayed for 6 hours on 3x10^4 ^{51}Cr-labeled P815 cells for 6 hours. Tumor-bearing mice had been injected with 3x10^4 P815 cells s.c. 24 days previously.

Table 4

Optimal Conditions for Induction of Syngeneic Anti-P815 CL

Concentration of IL2

IL2 (units/ml)	KA x 10^8
0	5
48	27
24	117
12	150
6	198
3	99

Normal DBA spleen cells, 1.25×10^6/ml, were cultured with 1.25×10^5 P815 cells for 5 days with the specified concentrations of IL2. This IL2 was maximally active on thymocyte proliferation and allogeneic CL generation at 3 units/ml.

Table 5

Efficacy of IL2 and P815 cells in the Generation of Syngeneic CL

P815	IL2	Normal DBA	KAx10^8 Tumor Bearing DBA Day 11	Day 21	Day 26	Normal CBA
Experiment A						
-	-	<5	23±5	30±7	15±3	<5
-	+	70±11	82±12	140±15	<5	30±6
+	-	<5	<5	15±4	<5	500±31
+	+	295±50	390±32	1230±14	230±14	4855±72
Experiment B						
-	-	<5	3±2	6±4	nd	<5
-	+	4±2	14±3	28±5	nd	<5
+	-	<5	2±4	3±4	nd	163±72
+	+	36±9	40±6	280±14	nd	1587±35

Spleen cells, 1.25×10^6, from DBA or CBA mice, as indicated, were cultured for 5 days. When present, IL2 was at optimal concentration (6units/ml), and P815 cells were at 6.25×10^4/ml. Tumor-bearing DBA mice had been injected with 1×10^3 P815 cells on the indicated number of days prior to use. Experiments A and B demonstrate the similarity in the pattern of results observed, despite day-to-day variations in the absolute levels of KA. With 1×10^4 assay cells/well, a KA value of 200×10^{-3} corresponds to 10% specific lysis after 5 hours of incubation at a 1:1 CL to tumor cell ratio.

cells and IL2 (Table 5). By day 25, 20% of nucleated cells in the spleen were morphologically indistinguishable from P815 cells. The CL response began to decline at this time. Even at this late stage of tumor growth, 1 to 3 days before death, significant CL activity could be generated from spleen cells by culture with IL2 and P815 cells (Table 5).

3.2.3 EFFICACY OF IL2 IN GENERATION OF SYNGENEIC CL

CL generated from cells from tumor-bearing mice by culture with IL2 and P815 cells were more active (KAx10^3 of 1230) than normal allogeneic CL generated by culture of CBA cells with (allogeneic) P815 cells alone (KAx10^3 of 500) (Table 5). CL generated from cells from P815 tumor-bearing DBA mice by culture with IL2 and P815 cells (KAx10^3 of 88) were more active than CL generated from cells from normal DBA mice by culture with only (semi-syngeneic, H2-d) S194 (KAx10^3 of 60) (Table 6). They were also more active than CL from DBA spleen cells cultured with only (allogeneic) EL4 difference (KAx10^3 of 22) (Table 6). Since both S194 tumors and EL4 tumors are rejected by normal DBA mice and P815 tumors are rejected by CBA mice, the level of activity generated by culture with IL2 and P815 cells should represent responses equivalent to those capable of tumor rejection.

Culture with added IL2 also increased the response of cells from normal and P815 tumor-bearing mice to minor (S194, KAx10^3 of 200 and 290, respectively) and major (EL4, KAx10^3 of 400 and 517, respectively) histocompatibility differences. Culture of cells from P815 tumor-bearing DBA mice generated more activity against P815 than did culture of cells from normal mice (KAx10^3 of 88 vs KAx10^3 of 44) in this experiment (see also table 3 and figure 2). Cultured cells from P815 tumor-bearing DBA mice did not express more activity against either S194 or EL4 than did cells from normal mice. Therefore, P815 tumor-bearing DBA mice contain a population of cells active against P815. The activity of these cells can be unmasked by culture with IL2 and P815 cells. They are as effective or more so than are CL directed against minor or major histocompatibility differences.

Table 6

Efficacy of IL2 and P815 in generating syngeneic CL

Comparison of minor and major H2 differences

Culture conditions		KA x 10^8	
Tumor	IL2	Normal DBA	Tumor-bearing DBA
-	-	6	< 5
-	+	9	16
P815	-	< 5	35
P815	+	44	88
-	-	24	30
-	+	62	50
S194	-	60	126
S194	+	200	290
-	-	< 5	10
-	+	8	25
EL4 (H2-B)	-	22	350
EL4	+	400	517

Spleen cells, 1.25×10^6, were cultured for 5 days with 1.25×10^3 of the tumor cells listed. Tumor-bearing mice had been injected with 1×10^4 P815 cells 14 days prior to use. DBA mice are H2-d. P815 tumor cells are H2-d. S194 cells are H2-d but Mls different from DBA mice. EL4 tumor cells are H2-b.

3.2.4 KINETICS OF GENERATION OF CL RESPONSE

Spleen cells from normal or tumor-bearing mice did not exhibit significant cytolytic activity when assayed immediately after being removed from the mouse (data not shown). Cytolytic activity first appeared 3 to 4 days after initiation of culture with P815 cells and IL2 (Fig. 2). Activity peaked at day 5 to 6 and declined rapidly thereafter. The maximal response of cells from tumor-bearing mice was 10 fold greater than that of cells from normal mice. There was little difference in the time course of CL generation from cells of normal or tumor-bearing mice. The kinetics were similar to those of generation of allogeneic CL (data not shown).

Culture of spleen cells from normal mice with IL2 alone generated cytolytic activity similar in magnitude and kinetics to that generated by culture of spleen cells with IL2 and P815 cells (Figure 2 and 3). This antigen non-specific activity arises perhaps slightly earlier than "classical" CL, but clearly not within the first 24 hours as would be expected of NK cells (263,264). Note that on day 5, when most of the assays were performed, the activity of cells cultured in IL2 alone was already decaying.

3.2.5 IL2 DEPENDENCE OF CL GENERATION FROM THYMOCYTES

Thymocytes from normal or tumor-bearing DBA mice cultured with syngeneic P815 cells and IL2 generated significant levels of CL (Table 7). They differed from spleen cells in requiring both IL2 and P815 cells to give measurable CL. Similar CL activity was observed with thymocytes from normal and tumor-bearing mice, suggesting that CL precursors were not increased in the thymus of tumor-bearing mice as they were in spleen (Table 5). The time course of CL generation from thymocytes was similar to that of spleen cells, except that the CL persisted slightly longer (data not shown).

3.2.6 SYNGENEIC CL ARE SPECIFIC FOR TUMOR ANTIGENS

CL generated from spleen cells from tumor-bearing DBA mice by culture with IL2 and P815 cells were active against P815 but not against normal DBA / 2J lymphoblasts or spleen cells (Table 8). Therefore, the CL recognized an antigen or antigens present on P815 cells but not on normal spleen cells. CBA (allogeneic) spleen cells cultured with P815 cells lysed all 3 targets, as expected (Table 8). Cultured DBA spleen cells lysed Con

Table 7

Effect of IL2 plus P815 Cells on DBA Thymus Cells

		KA x 10^8			
			Tumor-bearing Mice		
P815	IL2	Normal DBA	Day 11	Day 21	Day 25
-	-	<5	<5	<5	<5
-	+	<5	<5	<5	<5
+	-	<5	<5	<5	<5
+	+	290	245	270	<5

DBA thymus cells, 1.25×10^6/ml, were cultured with 6×10^4 P815 cells for 5 days. Assay of killing activity was for 4 hours on 5×10^5 ^{51}Cr-labeled P815 cells. Tumor-bearing mice were injected with 1×10^3 metastatic P815 cells s.c. the stated number of days prior to use. Twenty-five days after the injection of P815 cells, approximately 20% of the nucleated spleen cells were morphologically indistinguishible from P815 cells. No P815 cells could be detected in the thymus. Mice died 27±2 days after being injected s.c. with P815 cells.

Table 8

Specificity of CL Induced with IL2 and P815

I. DBA mice with DBA Targets			
	KA x 10^8		
Assayed On	Normal DBA	Spleen Cells from Tumor-bearing DBA	Normal CBA
P815	25±5	285±14	890±17
DBA Con A Blasts	<5	9±2	195±12
DBA Spleen Cells	<5	<5	36±4

Spleen cells, 1.25×10^6/ml, and 6×10^4 P815 cells/ml were cultured with IL2 for 5 days. Assay was for 5 hours on 5×10^4 of the indicated ^{51}Cr-labeled target cells for 5 hours. Con A blasts were prepared by culturing 3×10^6 spleen cells/ml with Con A for 48 hours. Normal spleen cells were labeled for 18 hours with ^{51}Cr as compared to 2 hours for P815 cells or Con A blasts. Spontaneous release for P815 cells was about 10%, for Con A blasts about 15% and for normal spleen cells about 20%. Tumor-bearing DBA mice were injected with 1×10^3 P815 cells s.c. 22 days prior to use.

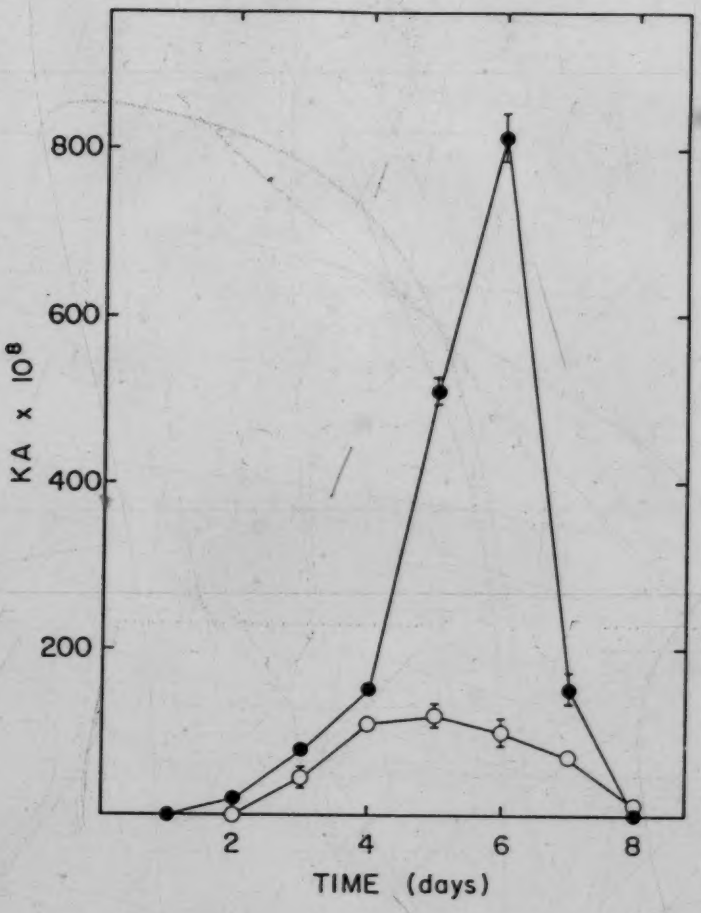

Figure 2: Time course of generation of CL activity by culture with IL2 and P815 cells

Spleen cells, 1.25×10^6/ml, from P815 tumor-bearing DBA mice (●), or normal DBA mice (○) were cultured with optimal IL2 and 6.25×10^4 P815 cells/ml. Each day cultures were assayed on 1×10^4 ^{51}Cr-labeled P815 cells/well. Spontaneous ^{51}Cr release was approximitely 10% on each day. Where bars are not present, the standard deviations are less than the circumference of the circles. Tumor-bearing mice had been injected with 1×10^4 P815 cells s.c. 18 days prior to the initiation of cultures.

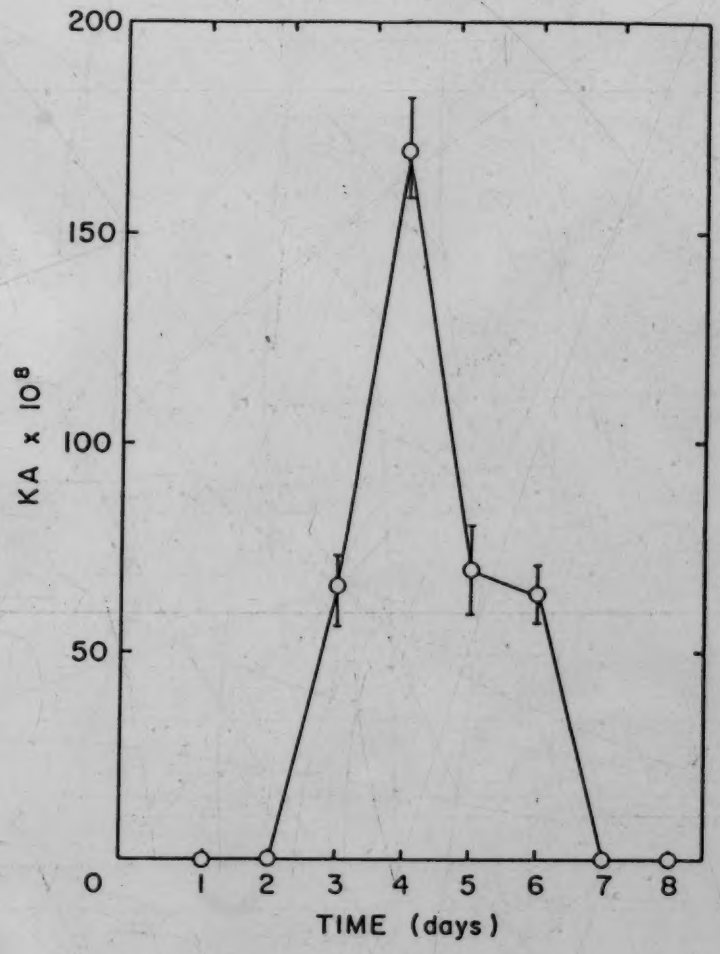

Figure 3: Time course of generation of CL with IL2

Spleen cells, 1.25×10^6/ml, from normal DBA mice were were cultured with optimal IL2. Each day, the cultures were assayed on 1×10^4 ^{51}Cr-labeled P815 cells/well. Spontaneous ^{51}Cr release was approximitely 10% on each day. Only normal mice are included in this figure because of the possibility of carry-over of antigen or tumor from tumor-bearing mice. The kinetics and magnitude was essentially the same for spleen cells from tumor-bearing mice (data not presented). Note the difference between the KA scale in this figure and in figure 2.

A blasts that had not been washed free of Con A (data not shown), presumably because Con A allows CL to kill target cells regardless of surface antigens (306). These results, taken together, suggest that the failure of syngeneic, tumor-specific CL to lyse normal, syngeneic cells was not due to their inherent resistance to lysis but was, instead, due to the syngeneic CL being specific for tumor antigens.

3.2.7 CL INDUCED IN THE ABSENCE OF ANTIGEN ARE ACTIVE AGAINST SEVERAL TUMOR LINES

CL generated from spleen cells of P815 tumor-bearing DBA mice by culture with IL2 but without antigen were active against a variety of tumor cell lines (Table 9). In addition to cytotoxicity against P815, a DBA/2J mastocytoma (H2-d) cytotoxicity was expressed against CaD2, a DBA/2J mammary adenocarcinoma (H2-d); S194, a BALB/c myeloma (H2-d); and MOPC 21/P3, a BALB/c myeloma (H2-d). A lower but significant degree of cytotoxicity was expressed against RI, a CBA/J lymphoma (H2-k); BW5147, an AKR lymphoma (H2-k); and perhaps L1210, a DBA/2J lymphocytic leukemia (H2-d). Cytotoxicity was also directed at EL4, a C57BL/6 T lymphoma (H2-b) (data not shown). Thus cytotoxicity was demonstrated against 3 syngeneic tumor lines, 2 semi-syngeneic BALB/c (H2 identical, but different genetic background from DBA) tumor lines, and 3 allogeneic (derived from H2-b and H2-k mice) tumor lines. The susceptibility of CaD2, a mammary carcinoma, suggested that lymphoid origin is not required for lysis. The only cell line not sensitive to lysis by the CL was 6140, which is a human lymphoblastoid line. Similar results were observed with spleen cells from normal mice (data not shown). The kinetics of generation of CL activity against P815 and other tumor lines were similar (data not shown).

Spleen cells from P815 tumor-bearing mice cultured with IL2 and P815 cells exhibited increased activity towards P815 compared with spleen cells cultured with IL2 alone. On the other hand, CL activity of spleen cells against cell lines other than P815 was not greatly affected by the presence of P815 cells in the sensitizing culture (Table 9). In summary, these results show that culture with P815 cells and IL2 generated a population of cells specific for P815. These cells were not activated in cultures without added P815 cells. In addition, a lower and less specific CL activity was generated by incubation with

Table 9

Specificity of CL Induced with IL2 and P815

IL2 Induced CL Lyse a Range of Tumor Cell lines

Assayed On		KAx 10^8 Culture Conditions			
		Medium	IL2	P815	P815 and IL2
P815	(DBA, H-2d)	90	115	49	1242
L1210	(DBA, H-2d)	<5	20	<5	6
CaD2	(DBA, H-2d)	45	75	24	116
S194	(Balb/c, H-2d)	45	350	21	640
P3	(Balb/c, H-2d)	<5	60	<5	140
R1	(CBA, H-2k)	<5	35	<5	24
BW5147	(AKR, H-2k)	<5	18	<5	23
6140	(Human)	<5	<5	<5	<5

Spleen cells from tumor-bearing mice were cultured as indicated for 5 days. Tumor-bearing mice had been injected with 1×10^3 P815 cells 21 days prior to use. Assay of killing activity was for 6 hours on 1×10^4 of the indicated cell lines.

IL2 alone.

3.2.8 CULTURE WITH IL2 AND TUMOR GENERATES TUMOR-SPECIFIC CL

The increased activity towards the sensitizing tumor of spleen cells cultured with tumor cells and IL2 was not restricted to the DBA-P815 system. Cells from normal or tumor-bearing DBA mice cultured with IL2 alone demonstrated significant activity towards P815, S194, and EL4 (Table 10). A consistant increase in lysis of the tumor cells added to the cultures was seen with spleen cells cultured with tumor cells and IL2 (Table 10). Compared to culture with IL2 alone, the increased activity in cultures with tumor and IL2 was approximately 4 fold for normal DBA spleen cells and P815 cells, 18 fold for tumor-bearing DBA spleen cells and P815 cells, 3.5 fold for normal DBA spleen cells and S194 cells and 7 fold for normal DBA spleen cells and EL4 cells.

A similar pattern was exhibited by CBA spleen cells cultured with syngeneic RI tumor cells (Table 11). Both P815 cells and L1210 cells were lysed with greater ease by cultured CBA spleen cells than were RI cells. The only increase in activity following culture with RI tumor cells and IL2 was for the sensitizing tumor, RI. The level of killing activity following culture with IL2 and RI cells increased 2.5 fold for spleen cells from normal mice and 60 fold for spleen cells from tumor-bearing mice. Once again spleen cells from syngeneic RI tumor-bearing mice demonstrated significantly greater activity towards RI cells following culture with IL2 and RI cells than did cells from normal mice ($KAx10^3$ of 360 and 55 respectively).

Culture of spleen cells with IL2-containing lymphokine preparations alone induced a population of cells capable of lysing a variety of tumor cells. Culture of spleen cells with tumor and IL2 induced an additional tumor-specific population directed against the sensitizing tumor cells. Spleen cells from tumor-bearing mice contained an increased amount of tumor specific activity. This activity could be unmasked by culture with IL2 and tumor.

Table 10

Specificity of CL Induced with IL2 and Tumor

Assayed on	DBA mice with various tumor types KA x 10^8 Culture Conditions			
	Medium	IL2	Tumor	Tumor and IL2
1. Normal DBA mice with P815 cells in culture				
P815*	18	29	7	130
S194	81	183	18	192
EL4	6	15	5	6
2. Tumor-bearing DBA mice with P815 cells in culture				
P815*	46	15	101	270
S194	90	128	91	230
EL4	27	75	15	88
3. Normal DBA mice with S194 cells in culture				
P815	18	29	21	60
S194*	81	183	180	600
EL4	6	15	39	211
4. Normal DBA mice with EL4 cells in culture				
P815	18	29	9	25
S194	81	183	24	204
EL4*	6	15	397	1068

Spleen cells, 1.25×10^6/ml, from normal or from P815 tumor-bearing mice were cultured for 5 days with 1.25×10^5 stimulating cells/ml of the indicated type. Assays were on 1×10^4 cells of the indicated type for 4.5 hours. The * represents those cultures with the same sensitizing and assay antigen. The tumor-bearing mice had been injected with 1×10^4 P815 cells/ml 14 days prior to use.

Table 11

Specificity of CL Induced with IL2 and Tumor

Assayed On	CBA mice with R1 tumor KA x 10^8 Culture Conditions: Medium	I12	Tumor	Tumor and I12
1. Normal CBA mice with R1 in culture				
R1	<5	21	7	55
P815	176	218	22	188
L1210	34	153	37	111
2. R1 tumor-bearing CBA mice with R1 in culture				
R1	<5	9	20	360
P815	<5	72	36	52
L1210	6	107	40	60

Culture conditions were identical to those in Table 10. CBA mice had been injected with 1×10^4 R1 cells 18 days prior to use.

3.2.9 COLD TARGET INHIBITION

The mechanism of recognition of several tumor lines by cells generated by culture with IL2 alone is not clear. To determine if clones of cells reactive to each cell type were involved or if clones of cells lysing several cell types were involved, cold target inhibition experiments were performed (Table 12). P815 and S194 were chosen as cell lines readily lysed by the IL2 generated cells. 6140 was chosen as a non-reactive target (Table 9). P815 cells and S194 cells were equally able to inhibit activity against either P815 cells or S194 cells suggesting that the same cell was lysing both S194 cells and P815 cells. 6140 cells did not significantly inhibit killing of either S194 cells or P815 cells. IL2 generated CL, therefore, seem to lyse populations of sensitive cells by similar or common cell surface antigens.

3.2.10 INTERFERON IN IL2 PREPARATIONS

The non-specific cytolytic activity of CL generated by culture with IL2 alone may have resulted from interferon effects on NK-like cells. Interferon activity in fraction 4 IL2 was therefore assessed (Table 13). IL2 contained 10 times less interferon activity assessed by plaque inhibition than IL2 activity assessed by thymocyte proliferation. The presence of interferon in fraction 4 IL2 was confirmed by K. Paucker. He determined, by antibody neutralization, that this was not "classical" or fibroblast interferon (K. Paucker, personal communication). The interferon present was probably immune interferon. Approximately 1/3 of the interferon present in fraction 3 IL2 moved with IL2 during DEAE chromatography (data not shown).

Neither DTT nor SDS treatment of fraction 4 IL2 significantly decreased interferon activity (Table 14). IL2 purified by chromatography over a Sephacryl S-300 column equilibrated with SDS retained IL2 activity but lost all significant interferon activity (Table 14). This IL2 was still fully active in thymocyte proliferation assays (40) and in generation of CL against syngeneic tumors when tumor was also present in the cultures (data not presented). This suggested that exogenous interferon was not necessary for CL generation in this system.

Table 12

Cold Target Inhibition

Assayed On	% Inhibition Competing Cell Type		
	S194	P815	6140
P815	66	65	0
S194	73	74	21

Spleen cells, 1.25×10^6/ml, from P815 tumor-bearing mice were cultured with IL2 for 6 days. Cultured cells were assayed on 5×10^4 ^{51}Cr-labeled target cells as indicated. Excess unlabeled targets, 10, 5, and 2 fold, of the specified type were added to each well. Data is presented for a 5:1 excess. Inhibition is expressed as (control-inhibited / control) x 100.

Table 13

Interferon Activity of IL2 (Fraction 4)

IL2 Units/ml	% Inhibition of Plaque Formation
40	66
20	55
10	30
NIH Interferon Units/ml	
4	95
2	85
1	70

L cells were cultured until forming a monolayer. Culture media was removed and cells were cultured overnight with IL2 or NIH standard interferon in 2 ml of media. Cells were then washed and infected with 2.5×10^4 plaque forming units of wild-type mengo virus (see Methods). Control plates had 88.5 plaques/plate. Inhibition is expressed as (control-inhibited/control) x 100.

Table 14

Separation of Interferon and IL2 Activity

IL2 Units/ml	Treatment	% Inhibition
40	none	66
20	none	55
10	none	30
40	DTT	65
20	DTT	52
10	DTT	18
40	SDS	65
20	SDS	52
10	SDS	35
	SDS Column Purified	
20 units		2.5

DTT and SDS-treated IL2 was kindly provided by B. Caplan (40). SDS column-purified IL2 was also provided by B. Caplan (see Methods) (40). Inhibition represents the inhibition of plaque formation by mengo virus on L cells as described in the previous table and in the Methods. Inhibition is expressed as (control-inhibited / control) x 100.

3.2.11 SYNGENEIC CL ARE THY 1⁺ AND LY 1⁺

Treatment of CL generated from spleen cells from normal DBA and CBA mice and spleen cells from tumor-bearing DBA mice with antibodies against Thy 1.1 or Ly 1.1 and complement removed more than 97% of the cytotoxic activity towards P815 cells (Table 15). Therefore, the P815-specific CL are probably "classical" cytotoxic T lymphocytes. The non-specific activity against S194 was somewhat less sensitive to Thy 1.1 and Ly 1.1. Only 90% of the activity was removed following treatment with antibodies against Thy 1.1 or Ly 1.1 (Table 16). Once again similar patterns were seen with spleen cells from CBA, DBA or tumor-bearing DBA mice. CL generated from spleens of tumor-bearing mice were somewhat less sensitive to Thy 1.1 and Ly 1.1 than were CL from normal mice.

3.2.12 IL2 GENERATED CL ARE NOT NYLON WOOL ADHERENT

Removal of cells adherent to nylon wool (156) did not significantly decrease the CL activity of spleen cells from P815 tumor-bearing mice against either P815 cells or S194 cells (Table 17). Adherent cells are not responsible for the lysis of either of the two tumor cell types.

3.2.13 GENERATION OF CL AGAINST OTHER SYNGENEIC TUMORS

The increased *in-vitro* cytolytic activity of spleen cells from normal and syngeneic tumor-bearing mice followng culture with tumor and IL2 was observed with L1210, a syngeneic DBA lymphoma (Table 18); CaD2, a syngeneic DBA mammary adenocarcinoma (Table 19); and RI, a syngeneic CBA lymphoma (Table 20). The responses to L1210 cells or RI cells were similar to the DBA-P815 system except that, in this experiment, culture with IL2 plus L1210 cells gave similar results to culture with IL2 alone. Cells from tumor-bearing mice consistantly demonstrated increased responses to syngeneic tumor compared to cells from normal mice. Cultures with IL2 and tumor had more CL activity than did cultures with tumor or IL2 alone.

Killing activity was consistantly lower in the CaD2 system. This may have been related more to the inherent resistance to lysis of CaD2 tumor rather than to decreased CL activity. In only half of the experiments did spleen cells from tumor-bearing mice demonstrate increased activity towards CaD2 cells following culture with IL2 and tumor

Table 15

IL2 and tumor generated CL are Thy 1.1^+ and Ly 1.1^+

Spleen Cells from	Treatment	KA x 10^8 Culture Conditions IL2	IL2 and P815
Normal DBA			
	none	15 ± 1	178 ± 1
	NMS+C	8 ± 4	61 ± 1
	Thy 1.1+C	< 2	< 2
	Ly 1.1+C	< 2	< 2
Tumor-bearing DBA			
	none	39 ± 2	786 ± 5
	NMS+C	27 ± 1	623 ± 4
	Thy 1.1+C	< 2	3 ± 1
	Ly 1.1+C	< 2	22 ± 1
Normal CBA			
	none	nd	436 ± 22
	NMS+C	nd	190 ± 11
	Thy 1.1+C	nd	< 2
	Ly 1.1+C	nd	< 2

Spleen cells from normal or tumor-bearing mice were cultured as indicated at 1.25×10^6 cells/ml in 10 ml cultures. Tumor-bearing mice had been injected with 1×10^4 P815 cells 19 days prior to use. Tumor-bearing mice had been injected with 1×10^3 P815 cells 20 days prior to use. After 5 days of culture, viable cells were harvested on ficoll-hypaque. The viable cells were incubated with either normal mouse serum (NMS), Thy 1.1 or Ly 1.1 antibodies and rabbit complement (C) as indicated (see Methods). Viability of the cultured DBA spleen cells following incubation with Thy 1.1 or Ly 1.1 antibodies and complement was 20 and 18%, respectively. Only 5 and 10% of the cultured CBA spleen cells were viable following treatment with Thy 1.1 and Ly 1.1 antibodies, respectively. Incubation with normal mouse serum and complement decreased the viability by only 10%. Assay of killing activity was for 4.5 hours on 1×10^4 P815 cells.

Table 16

IL2 generated cross-reactive CL are mainly Thy 1.1^+ and Ly 1.1^+

Spleen Cells from	Treatment	KA x 10^8 Culture Conditions	
		IL2	IL2 + P815
Normal DBA	None	78	205
	NMS+C	67	248
	Thy 1.1+C	0	39
	Ly 1.1+C	28	24
Tumor-bearing DBA	None	242	544
	NMS+C	211	384
	Thy 1.1+C	7	28
	Ly 1.1+C	9	76
CBA/J	None	nd	441
	NMS+C	nd	398
	Thy 1.1+C	nd	15
	Ly 1.1+C	nd	46

Culture conditions and incubations were identical to those in the previous table. Cultured cells were assayed on 1×10^4 S194 cells for 4.5 hours.

Table 17

Nylon Wool Treatment of CL

		KA x 10^8			
		Culture Conditions			
Assayed On	Treatment	Medium	IL2	P815	P815 and IL2
P815	none	<5	145	70	800
	nylon wool	<5	200	10	750
S194	none	<5	115	<5	165
	nylon wool	45	130	<5	115

Spleen cells, 1.25×10^6/ml from tumor-bearing mice were cultured for 5 days as described above. IL2 was added at 6 units/ml and P815 cells were at 6×10^4/ml. Tumor-bearing mice had been injected s.c. with 1×10^4 P815 21 days previously. Cultured CL, 10^7, were applied to a 20 ml nylon wool column to remove adherent cells as described in the Methods. About 50% of the cells adhered to the column. Assay was for 4.5 hours on 1×10^3 ^{51}Cr-labeled cells.

Table 18

IL2 in DBA response to syngeneic L1210

Culture Conditions		KA x 10^8	
L1210	IL2	Normal DBA	L1210 Tumor-bearing DBA
Experiment A			
-	-	<5	11
-	+	14	91
+	-	<5	22
+	+	8	127
Experiment B			
-	-	<5	17
-	+	<5	27
+	-	<5	<5
+	+	18	38

Spleen cells, 1.25×10^6/ml, from normal or L1210 tumor-bearing DBA mice were cultured with IL2 and L1210 cells as indicated. Assay was on 3×10^4 ^{51}Cr-labeled L1210 cells. Tumor-bearing mice were injected with 1×10^4 L1210 cells 18 days prior to use.

Table 19

IL2 in DBA Response to Syngeneic CaD2

Culture Conditions		KA x 10^8	
CaD2	IL2	Normal DBA	CaD2 Tumor-bearing DBA
-	-	<5	<5
-	+	<5	104
+	-	<5	<5
+	+	20	272

Culture was exactly the same as in Table 18 except that CaD2 tumor cells were used. Tumor-bearing mice had been injected s.c. with 1×10^4 CaD2 cells 14 days prior to use. Assay was for 6 hours on 1×10^4 ^{51}Cr-labeled CaD2 cells.

Table 20

IL2 in CBA Response to Syngeneic RI

Culture Conditions		KA x 10^8	
RI	IL2	Normal CBA	RI Tumor-bearing CBA
-	-	<5	63
-	+	68	140
+	-	64	430
+	+	88	898

Culture conditions were exactly the same as in the previous two experiments except that CBA mice and syngeneic RI tumor cells were used.

cells. Anti-CaD2 activity was totally lacking unless IL2 was added to the cultures (Table 19).

3.2.14 CONTINUOUS LINES OF SYNGENEIC ANTI-P815 CL

Continuous lines of CL were developed to determine the cellular characteristics, the origin of cross reactivity and the *in vivo* activity of CL free of other cell types. Spleen cells from P815 tumor-bearing mice were cultured with and without added P815 cells (Tables 21 and 22, respectively). Irradiated P815 cells were added only to the initiating cultures. IL2 was added to all cultures. Significantly greater activity against the P815 tumor cells was noted in those cultures initiated with P815 cells present. Both continuous lines mediated significant activity against syngeneic targets, P815 and CaD2; semi-syngeneic targets, S194; and allogeneic targets, EL4 and RI. The observed anti-S194 activity of the cell line was as high as the anti-P815 activity. Since S194 cells were ten times more senstive to lysis than P815 cells (data not shown), the level of killing probably represents more activity specific to P815.

3.2.14.1 CELLULAR CHARACTERISTICS OF CL CLONES

Morphology of the cell lines was similar to that previously described for CL directed against alloantigens (24). The cells were large, irregular, vacuolated and adherent. The CL retained a requirement for exogenous IL2 throughout the culture period. Cytolytic activity varied with time and the condition of the cells. The cells were repeatedly demonstrated to express Thy 1 (data not shown). On the one occasion that they were tested, they had Ly 1 on their surface (data not shown).

3.2.14.2 NON-SPECIFIC ACTIVITY OF CL CLONES

Spleen cells cultured from P815 tumor-bearing mice were expanded in medium containing IL2 and subsequently cloned at 1 cell/well (Table 23). Growing cells were present in 6 out of 96 wels set at 1 cell/well. These CL clones expressed significant activity against P815 cells, S194 cells, EL4 cells and RI cells (Table 23). The activity was similar to that of the original cell line (Table 22) and to short term cultures from tumor-bearing mice (Table 9 and 10). It appears, therefore, that the non-specific activity of cells cultured in IL2 without added antigen rests in clones of cells able to lyse several

Table 21

Continuous Lines of Syngeneic Anti-P815 CL

Culture with Antigen

Day Assayed	Target KA x 10^8				
	P815	S194	CaD_2	EL_4	R1
Day 5	100 (90)				
Day 9	714		100		
Day 32	277 (365)	227 (308)		74 (36)	69 (30)
Day 67	644	752			256

Spleen cells from tumor-bearing DBA mice were cultured at $1x10^6$ cells/ml with $5x10^4$ P815 cells and IL2. Cells were diluted to $1x10^4$ cells/ml every 4 to 5 days. Fresh medium with IL2 but without P815 cells was added every 2 to 3 days. The activity of the cell line was assayed by limiting dilution on $1x10^4$ ^{51}Cr-labeled target cells/well. Values in brackets represent killing which occured when 10 ug/ml Con A was added to the assay wells. DBA mice had been injected with $1x10^4$ P815 cells 19 days prior to use.

Table 22

Continuous Lines of Syngeneic Anti-P815 CL

Culture without antigen

Day Assayed	Target KA x 10^8			
	P815	S194	EL_4	RI
Day 22	16 (331)		155 (471)	39 (383)
Day 30	29 (210)		314 (500)	65 (248)
Day 65	103 (241)			
Day 180	7 (157)	439 (837)	42 (258)	60 (371)

Cultures were established as in the previous experiment except that the spleen cells were from tumor-bearing mice and P815 cells were not added to the cultures. Tumor-bearing mice had been injected with $1x10^4$ P815 cells 18 days prior to use. Values in brackets represents killing activity with Con A added at 10 ugm/ml in the assay.

Table 23

Continuous Lines of Syngeneic Anti P815 CL

Culture Without Antigen

Selected Clones

Clones	Day Assayed	Target KA x 10^8 P815	S194	RI	EL4
1.2	150	0 (34)		11 (10)	0 (113)
1.2	180	40 (512)	1919 (1939)	0 (389)	623 (1485)
1.3.1	180	67 (223)	1044 (1222)	11 (340)	323 (834)
1.2	200	0 (118)	585 (1062)	14 (344)	7 (306)
1.2	300	65 (462)	2941 (1066)	0 (286)	95 (285)
1.3.1	300	10 (160)	566 (519)	0 (36)	19 (63)
1.2	380	74	115	0	
1.3.1	380	18	86	0	

Cells from the previous experiment were cloned by limiting dilution on day 40. Clones were selected from plates established at 1 cell/well. Only 6 clones grew at 1 cell/well. Clone 1.2 was from this group.

Clone 1.3.1 was cloned from the parent population through sequential clonings at 1 cell and then 0.5 cells/well. Values in brackets represents killing activity with 10 ug/ml Con A added to the assay wells.

tumor lines. Similar cross-reactivity has been demonstrated with other continuous cell lines (24,79,99,331).

3.2.15 APPLICATION TO HUMAN SYSTEMS

3.2.15.1 PREPARATION OF HUMAN IL2

Because of the reported inability of murine IL2 to stimulate human cells (105), conditions for production of human IL2 were determined. Culture of mouse thymocytes at low cell concentrations with IL2 and Con A provided a sensitive assay for human IL2 (data not shown).

Human peripheral lymphocytes, 2×10^6, from two donors were pooled and cultured with 1×10^6/ml Daudi cells or 6140 cells and 24 ug/ml Con A. The supernatants contained between 10 and 20 units IL2/ml. These supernatents were freeze dried, dissolved in distilled water and desalted by passage over a G-10 column. The resulting solution was then run over a Sephadex G-100 column (Figure 4). Human IL2 assayed on mouse thymocytes demonstrated a broad peak of activity centered near that of cytochrome c (MW 12,900) which is slightly less than that of other reports (106).

Human IL2 prepared with high concentrations of Con A was inhibitory to CL responses unless the Con A was removed by passage over a Sephadex G-10 or G-100 column . Alternatively, the Con A binding could be blocked by addition of alpha-methylmannoside (data not shown).

3.2.15.2 MURINE IL2 IS EFFECTIVE IN GENERATING HUMAN CL

Because large amounts of human IL2 were difficult to obtain, the efficacy of murine IL2 in augmenting human CL responses was assayed. Relatively impure (fraction 4) IL2 increased the generation of CL against the human tumor line, 6140 (Table 24). Culture with murine IL2 generated a low level of activity against 6140 cells in the absence of antigen but significantly more activity was observed following culture with tumor plus IL2.

A human ovarian carcinoma cell line, A.F., proved to be very useful for the assay of human IL2 activity. Cells from donor G.M. did not respond to this cell line without culture with added IL2. Culture with exogenous IL2 of either mouse or human origin resulted in CL generation (Table 25). Peripheral lymphocytes from other donors (B.D, C.G

Table 24

Efficacy of Mouse IL2 in Generation of Human CL

Donor	Culture Conditions	Response KA x 10^8 Exp. 1	Exp. 2
G.M.	Medium alone	<5	0
	Mouse IL2	10	7
	Tumor	130	67
	Tumor & Mouse IL2	920	120

Peripheral blood lymphocytes, 1.5×10^6/ml, were cultured for 7 days with 7.5×10^4 6140 cells. Assay of killing activity was for 4.5 hours on 1×10^4 ^{51}Cr-labeled 6140 cells.

Table 25

Response of Human Peripheral Lymphocytes to Human Ovarian Carcinoma Line A.F.

Culture Conditions	Response KA x 10^8 Donors G.M.	G.M.	B.D.	B.D.	B.C.	C.G.
Medium	5(90)	<5	N.D.	N.D.	N.D.	5
Human IL2	N.D.	<5	N.D.	N.D.	N.D.	41
Mouse IL2	40(160)	<5	N.D.	N.D.	N.D.	N.D.
Tumor	5(90)	<5	6(11)	<5(<5)	54	29
Tumor & Human IL2	N.D.	60	N.D.	N.D.	282	122
Tumor & Mouse IL2	130(210)	86	71(65)	52(48)	N.D.	N.D.

Peripheral blood lymphocytes, 1.5×10^6/ml, were cultured as described. Where present, A.F. tumor cells were at 7.5×10^4 cells/ml. Human IL2 was fraction 3 at 1/10 dilution. Mouse IL2 was fraction 4 produced from spleen cells. Mouse IL2 was used at 6 units/ml. All cultures were for 7 days (optimal time, data not presented). Assays were for 4.5 hours on 1×10^4 ^{51}Cr-labeled A.F. tumor cells. Data in brackets represents the killing activity when 20 ug/ml Con A was added to the assay.

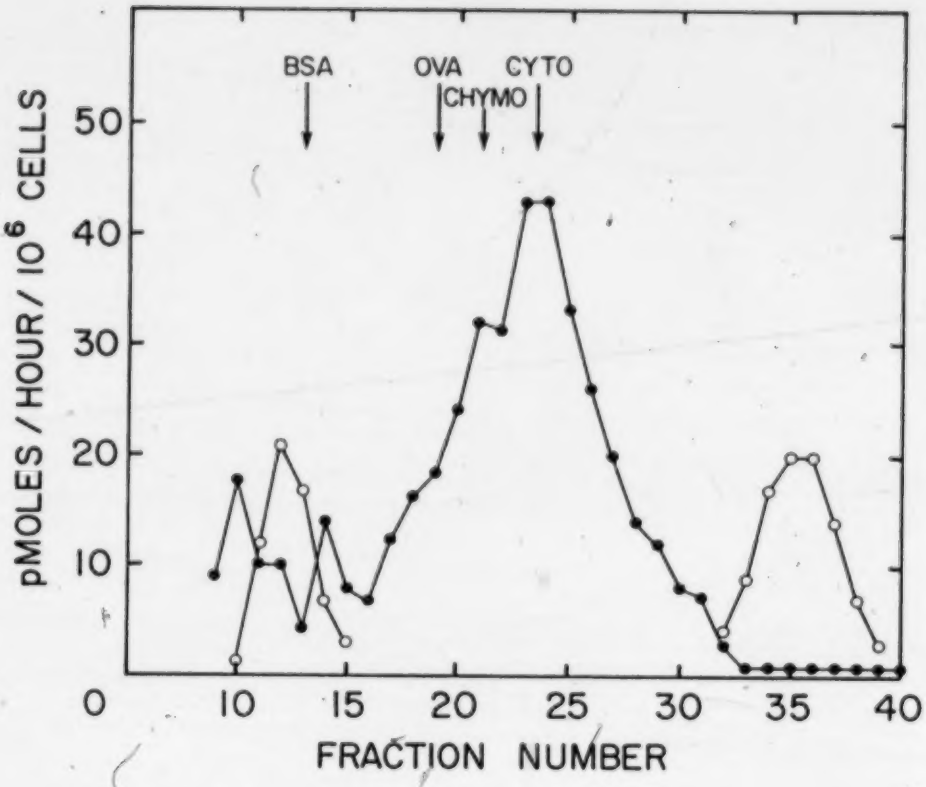

Figure 4: G-100 chromatography of human IL2.
Human IL2, made by culturing 2×10^6 peripheral lymphocytes/ml from 2 donors in 40 ml of medium with 24 ugm/ml Con A and 2×10^6 Daudi cells/ml for 48 hours, was concentrated by freeze drying and redisolved in 1 ml of medium. This was desalted by chromatography over a G-10 column. One half ml of this sample was layered onto a calibrated G-100 column. The column had been calibrated with bovine serum albumin (BSA) MW=43,000, ovalbumin (Ova) MW=43,000, chymotrypsinogen A (Chymo) MW=25,000, and Cytochrome C (Cyto) MW=12,900. Totally excluded and totally included values were determined with ^{3}H-labeled Escherichia coli DNA and ^{14}C labeled thymidine (0), respectively. One ml fractions were collected and assayed at 1/10 dilution on 5×10^5 thymocytes cultured for 72 hours with 4 ugm/ml Con A. Fraction 4 murine IL2 gave a maximum rate of proliferation of 83 picomoles/hr/10^6 cell. Proliferation was measured by adding ^{3}H-thymidine for the final four hours of culturing. Cells were harvested and counted on a Beckman scintillation counter (see methods). Human IL2 therefore moves with an apparent MW of 10,000 to 20,000.

and B.C) responded to the tumor in the absence of IL2 although the response of B.D. was consistantly low (Table 25). Responses of all donors were increased by culture with murine IL2 or with human IL2-containing preparations.

The specificity of the cultured CL was tested by addition of Con A (306). The CL activity following culture with IL2 alone or with IL2 and tumor was, generally, similar to the CL activity observed in the presence of Con A (Table 25). This suggests that most of the CL activity induced by culture with IL2 and tumor cells was specific to the sensitizing tumor.

Cells from B.D., initially cultured with the A.F. line, were cultured in crude human IL2 with alpha-methylmannoside for 30 days. There was no loss of cytolytic activity. The cell line increased in number by a factor of about 10^3 during this time (data not shown).

3.2.15.3 RESPONSE OF HUMAN PATIENTS TO AUTOLOGOUS TUMOR

Once it was established that both murine and human IL2 could augment human CL responses, the effect of IL2 on the response of cells from tumor patients to autologous tumor was determined (Table 26). Three patients with malignant serous cystadenocarcinomas (M.Z., A.F., and M.L.) and one with a benign serous cystadenoma (G.F.) were assessed. The diagnosis was not known at the time of assessment. Following culture with tumor and IL2, cells from M.Z., on one occasion, and A.F., on two different occasions, showed significant responses to autologous tumor. Cells from A.F. showed an increased response in cultures with IL2 alone. The pattern of non-specific reactivity illustrated with Con A was similar to that of allogeneic responses (Table 24 and 25).

Cells from M.Z. and A.F. were cultured continuously for 18 and 30 days, respectively, with a potential increase in cell number of about 10^3. No significant change in cytolytic activity per cell was noted. Both cell lines remained dependent on exogenous IL2. Both cell lines ceased to grow shortly thereafter and could not be recovered by addition of IL2 or addition of frozen tumor cells.

Peripheral lymphocytes from G.F. and M.L. did not respond to tumor in the presence or absence of IL2. Since allogeneic responses to these tumor cells (positive controls) were not assessed, the lack of response may have resulted from technical problems or from intrinsic resistance of the tumor cells to lysis. G.F. had a benign tumor, and tumor lysis was not expected. M.L. died three days after the cultures were

Table 26

Response of Patients to Autologous Tumor

Culture Conditions	Response KA x 10^8 Patient M.Z.	A.F.	A.F.	G.F.	M.L.
Medium	<5	8(80)	<5	<5	<5
Mouse IL2	<5	16(60)	N.D.	<5	<5
Human IL2	N.D.	N.D.	20	N.D.	N.D.
Tumor	<5	26(10)	<5	<5	<5
Tumor & Mouse IL2	29	160(130)	N.D.	<5	<5
Tumor & Human IL2	N.D.	N.D.	42	N.D.	N.D.

Peripheral blood lymphocytes were cultured at 1.5×10^6/ml with 7.5×10^4 irradiated autologous tumor cells/ml for 7 days. M.Z., A.F. and M.L. had serous cystadenocarcinomas. G.F. had a benign serous cystadenoma. All patients were from 65-75 years old. All assays were on freeze-stored autologous tumor cells. The numbers in brackets represent the killing activity following the addition of 20 ug/ml Con A to the assay wells.

established. A generalized hyporeactivity may have accompanied her late stage of disease.

3.3 DISCUSSION

3.3.1 TUMOR MODEL SYSTEMS

The murine tumor model systems were similar to some human tumor systems. P815 grew i.p. much like epithelial ovarian carcinoma with ascitic tumor growth followed by late systemic spread. P815, L1210, and RI s.c. grew rapidly as a solid tumor mass. These tumors metastasized early with death resulting from the metastases. Surgical removal of the primary tumor did not affect survival times.

3.3.2 CL INDUCTION IN TUMOR-BEARING MICE

Following injection of transplantable tumors, mice can exhibit a transient *in vivo* response to the tumors. Tumor size reaches a plateau but several days later continues to grow unabated (20,308). Irradiated mice do not develop this transient *in vivo* response. Irradiated mice demonstrate more rapid tumor growth and earlier metastases (20). Despite inability to control autologous tumor growth, spleen cells from mice with progressive tumors can prevent tumor growth in adoptive transfer assays demonstrating an *in vivo* anti-tumor response (61,322). Similarily, a second tumor graft can fail to take in tumor-bearing animals due to "concomitant" immunity (61,74,75,204). Concomitant immunity is defined as the ability of a mouse with a tumor growing at one site to prevent the growth of an injection of tumor cells at another site (19,20,75).

The waning of the *in vivo* cytolytic response is related to an increase in tumor-specific suppressor cells (19,74,308,309). Progressive tumor growth and inability to adoptively transfer immunity late in tumor growth indirectly suggests the presence of suppressor mechanisms (129,223). Direct evidence for suppressor cells is provided by blockade of the transfer of adoptive immunity into normal mice. This blocking activity is present in cells from mice with established tumors (20,74). Suppressor cells induced by culture can prevent the transfer of adoptive immunity by cells from immune mice (125).

Anti-tumor activity can be demonstrated by the generation of CL from tumor-bearing animals *in vitro* (Table 5) (see also 171,278,308,309). The ability to elicit cytolytic activity generally wanes as tumor growth progresses due to the presence of suppressor T cells (278,309). These suppressor T cells may produce a soluble inhibitor (186,278,309). Culture with IL2 and tumor results in CL generation from mice with established P815, RI, L1210, and CaD2 tumors (Table 5, 18, 19, and 20) and humans with ovarian tumors (Table 26) despite any suppressive effects which might be active *in vitro* (308) or *in vivo* (19,20).

Because IL2 can override the effect of the suppressor cell, at least *in vitro*, the suppressor cell may be preventing IL2 release. Suppressor cells generated in cultures can prevent IL2 release from stimulated cells (131,170,224). This suggests that in tumor-bearing mice, CL induction may be prevented by a suppressor cell blocking IL2 release.

3.3.3 INDUCTION OF CL ACTIVITY WITH IL2

IL2 or IL2-containing lymphokine preparations have been used by other investigators to increase responses to both allogeneic and syngeneic tumor cells. Culture in IL2 increases responses to allogeneic tumor cells enabling long term growth and cloning of these CL (99,100). Culture with IL2 significantly increases the CL activity of cells from tumor-immune mice (50,51,76,276) and tumor-immune rats (89,90). Culture with IL2 expands the numbers of cells along with increasing their activity. Culture with IL2 can increase the cytolytic activity of cells from normal mice against syngeneic tumors (97,344).

Growth in IL2 allows expression of cytolytic activity from cells from tumor-bearing mice (344,361) and from cells from tumor-bearing humans (190,330,332,364). CL reactive against several cancer types including lung, melanoma, breast, leukemia, osteosarcoma, lymphoma, hypernephroma and ovarian carcinoma have been generated (Table 25) (190,330,332,364).

The IL2 preparation used in most of our experiments was known to contain interferon, IL3, and CSF-GM (data not presented). These and other soluble factors may have played a role in the generation of CL. More purified preparations and effective

blocking antibodies will be necessary to ascertain the specific requirements for CL generation from tumor-bearing animals. IL2 purified by passage over a Sephacryl S-300 column equilibrated in SDS lacked interferon activity. This preparation was still effective in the generation of tumor-specific CL. This suggests that exogenous interferon is not necessary for generation of tumor-specific CL from spleen cells from tumor-bearing mice. This does not preclude a role for endogenous interferon.

3.3.4 TUMOR-BEARING MICE CONTAIN A POPULATION OF ACTIVATED CELLS

Direct evidence for a 10-fold to 20-fold increase in CL precursors has been demonstrated in mice with spontaneously regressing viral tumors (195). The frequency of CL precursors to the syngeneic tumor MBL-2 was 1/7,400 in normal spleen cells, 1/475 in spleen cells from mice with spontaneously regressing tumors, and as high as 1/98 in the tumor itself (195).

In the present work, significantly greater cytolytic activity could be elicited by culture with IL2 and tumor cells from spleen cells of tumor-bearing mice than from spleen cells from normal mice. This suggested that tumor-bearing mice contain a population of potential cytotoxic cells (Tables 5, 18, 19, and 20). This activated population was not present in the thymus (Table 7). Because these activated cells were responsive to IL2 *in vitro*, part of the inability of lymphocytes from tumor-bearing mice to exhibit cytotoxicity *in vivo* or directly on *in vitro* assay could be due to lack of IL2 release *in vivo*.

As culture with both IL2 and P815 cells was required to obtain optimal responses, the lack of an *in vivo* cytolytic anti-tumor response is not purely due to a lack of IL2 effect. Although there may be a block of IL2 production, there is probably also a block in the induction of CL prior to the expression of IL2 receptors.

3.3.5 SPECIFIC CYTOLYTIC ACTIVITY

The tumor-specific cytolytic activity generated from normal or tumor-bearing mice and humans by culture with IL2 and tumor cells is probably due to "classical" cytotoxic T lymphocytes. The anti-P815 CL were Thy 1 and Ly 1 positive (Table 15) (cytotoxic T lymphocytes in DBA mice are normally Ly 1 positive), were present with a time course appropriate for cytotoxic T lymphocytes (Figure 3), were active in standard short-term

^{51}Cr release assays, were found primarily in spleen, and were not adherent to nylon wool (Table 17). They were specific in that normal DBA cells were not lysed by CL directed against syngeneic P815 cells (Table 8) and in that a specific anti-tumor population was induced by culture with IL2 and tumor cells (Table 9,10,11). These are all characteristics of "classical" cytotoxic T lymphocytes.

3.3.6 NON-SPECIFIC CYTOLYTIC ACTIVITY

The situation is somewhat less clear for the lower, non-specific activity against several tumor lines that was generated by culture with IL2 in the absence of added tumor antigen. Although non-specific activity appeared in culture with a time course appropriate for CL (Figure 4), several tumor lines were lysed by cells from initial cultures (Table 9), by cells from continuous lines (Table 22) and by cells from cloned lines (Table 23). It was, therefore, thought that the effector cells might be a form of NK cells. Over 80% of the non-specific cytolytic activity was removed by monclonal antibodies against Thy 1 and 90% of the activity was removed by monoclonal antibodies against Ly 1 (Table 16). Although these antigens are not present on NK cells taken directly from the mouse, they are present in significant amounts on cultured NK cells (163,247). This prevents any differentiation between NK activity and CL activity by these surface phenotypic markers alone.

When cells from normal or tumor-bearing mice were cultured and cloned in IL2 without antigen, the clones demonstrated cross-reactivity patterns similar to the original uncloned cultures (163). These cells were noted to be Thy 1^+ always, Ly 1^+ occasionally, and Ly 2^+ most of the time. The cell lines were positive for asialo GM I, a marker normally present on NK cells but not on CL (163). Following culture with IL2, cells expressing CL or NK-like activity could not be separated on the basis of cell surface phenotype.

Interferon may play a role in the generation of these non-specific cytolytic cells. IL2 preparations devoid of interferon activity generate fewer of the non-specific cytolytic clones in limiting dilution experiments than do interferon-containing preparations (H.S. Teh, personal communication). IL2 preparations, without detectable interferon activity, can induce interferon production from CL (81,82,83,168). Therefore, unless interferon is specifically blocked, its action cannot be ruled out.

Two cell lines normally resistant to NK activity (P815 and EL4) (163) were sensitive to the cytolytic activity generated by culture with IL2 alone (Table 9 and 22). The ability of NK cell lines expanded in IL2 to lyse cells normally resistant to NK activity has been confirmed with cloned cells (163). Two types of clones have been isolated by Kedar (163). The first lyses only "classical" NK sensitive lines, whereas the other lyses both NK sensitive and resistant cell lines (163). The IL2-generated, murine cytolytic cells did not lyse human cell lines (Table 9) (163).

The mutual blocking of cytolytic activity against P815 cells and S194 cells by both cell types and not by the human line, 6140, suggests that the cytolytic cells recognize a structure present on both P815 cells and S194 cells (Table 12). The ability of clones of NK cells to lyse a range of cells also suggest that they recognize a structure present on the surface of all NK-sensitive cells (Table 23) (166).

IL2, in the absence of added antigen, generates clones of activated cells which are much less specific than those generated with IL2 and antigen. The non-specific reactivity we observed, arises from a population of NK-like CL or a population of NK cells. Further experiments will be necessary to distinguish between the two possibilities.

3.3.7 SUMMARY

Tumor-bearing mice contain two populations of cells sensitive to IL2 or other factors present in the IL2 preparations. The first population, sensitive to IL2 alone, is non-specific, lysing a range of tumor lines including the tumor present in the mice. This population is present in spleen but absent from the thymus. The second population, sensitive to expansion by culture with IL2 and tumor, is specific. It lyses only the sensitizing tumor. The specific population is increased in frequency in the spleens of tumor-bearing mice. It appears, therefore, that IL2 can expand clones of two types of cytotoxic cells, one specific for and limited to the tumor, the other able to kill a number of tumor targets.

3.3.8 GENERATION OF HUMAN ANTI-TUMOR CL

The optimal conditions found for producing human IL2 were co-culture of cells from several donors with the lymphoblastoid line, Daudi, in the presence of Con A. These conditions were similar to those reported previously (26,106). Allogeneic antigens on the co-cultured cells and Con A supply a strong stimulatory signal. Daudi decreases the activity of prostaglandin released by macrophages (255). As mentioned earlier, this prostaglandin could decrease IL2 release (53,255). Human IL2 preparations must be depleted of Con A to be effective in increasing the activity of human CL (data not shown).

Although murine IL2 does not stimulate the continuous proliferation of human CL (291), it can increase the CL activity in cultures of normal human cells with the human lymphoblastoid line, 6140, and the human ovarian adenocarcinoma line, A.F. (Tables 23,24,25). The reason for this discrepancy with previous reports is not clear. The difference in the assay systems, continuous proliferation vs CL generation, may be important. Alternatively, since the IL2 preparations used were relatively impure, factors other than IL2 may have resulted in the proliferation of the human CL.

Peripheral blood lymphocytes of two out of three ovarian carcinoma patients studied had an autologous anti-tumor reactivity which could be demonstrated by culture with human IL2 or murine IL2 and autologous tumor (Table 25). The non-responding patient died soon after assessment and the lack of a response may have been related to her generalized debility. The patient with a benign tumor did not respond to her tumor.

Short term cell lines reactive to autologous tumor could be generated by culture with human IL2. These cell lines could be expanded at least a thousand fold without loss of cytolytic activity.

The CL activity generated from peripheral blood cells against autologous tumors was comparable to that of normal donors against the human tumor line, A.F. These preliminary results suggest that some tumor-bearing patients have a population of cells capable of becoming CL upon culture with IL2 and tumor.

3.3.9 EFFICACY OF CL INDUCTION

Mice are normally able to reject tumors expressing minor or major histocompatibility differences probably through a mechanism involving the generation of CL (328). Additionally, mice can be rendered tumor-immune by various treatments which might also involve the generation of CL. If CL as active as those preventing growth of allogeneic tumors or those in mice immune to syngeneic tumor can be generated from tumor-bearing mice, the possibility of effective cancer therapy by infusion of the activated CL may be possible. Therefore, the efficacy of our system for generating CL from tumor-bearing mice by culture with IL2 and tumor will be compared to other systems involving allogeneic tumors, tumor-immune mice, normal mice and tumor-bearing mice.

3.3.9.1 COMPARISON TO ALLOGENEIC TUMORS

Culture of cells from tumor-bearing mice and humans with IL2 and syngeneic tumor cells allowed the generation of highly active cytolytic activity (Table 5,6,19,20,21, and 25). This cytolytic activity was more active than that generated by allogeneic cells against the same tumor (Table 5). The cytolytic activity against syngeneic tumor was greater than that of the same mice against minor or major histocompatibility differences (Table 6). Since these mice were able to reject tumors with minor or major histocompatibility differences and CL can play a role in tumor prevention (328), the anti-syngeneic tumor response generated by culture of cells from tumor-bearing mice with IL2 and syngeneic tumor may be capable of controlling *in vivo* tumor growth (328).

Culture with exogenous IL2 not only increased syngeneic anti-tumor responses; it also induced a significant increase in responses against minor and major histocompatibility differences (Table 5, 6). IL2 production may, therefore, be one of the limiting factors in CL generation to syngeneic tumor antigens and also to some alloantigens.

3.3.9.2 COMPARISON TO GENERATION OF CL FROM IMMUNE MICE

Mice can be rendered immune to some syngeneic tumors by various techniques. Injection of irradiated (51,89,90,287) or mitomycin C treated (267) syngeneic tumor cells can result in tumor-immune mice. Cross-reactive allogeneic tumor cells can occasionally induce immunity to syngeneic tumors (99). Surgical removal of some tumors results in tumor immunity (3,118,119). Some tumors grow progressively when injected into one

site (usually i.p.) but result in tumor immunity when injected into other sites (usually intradermal) (3,45,151). Injection of Corynebacterium parvum early in tumor growth can also give tumor-immune mice (19). This inducible immunity along with the concomitant immunity described earlier (20,61) establishes the immunogenicity of some transplantable tumors.

Tumor-immune mice contain populations of cells which became effective CL upon culture with tumor alone. Presumably tumor-immune mice are resistant to further tumor challenge due to an ability to generate effective CL *in vivo* upon tumor challenge (86,328). Cytotoxic responses of cells from tumor-immune mice measured either directly from the mice or following culture with tumor cells were either quite poor (44,45,87,88,89,90,151) or comparable to those obtained with culture of spleen cells from tumor-bearing mice with IL2 and tumor (Table 5, 18, 19, 20) (3,37,118,119,245,287). Therefore, it appears that culture of spleen cells from tumor-bearing mice with IL2 and tumor generates a population of CL at least as active as the CL which can be generated from tumor-immune mice.

3.3.9.3 COMPARISON TO GENERATION OF CL FROM NORMAL MICE

A much lower cytolytic activity against P815 could be elicited by culture of spleen cells from normal mice compared to culture of spleen cells from P815 tumor-bearing mice (Table 5). It has generally proven difficult to generate cytotoxic lymphocytes active against syngeneic tumors from cells from normal mice (138,267). The responses *in vitro* have been low compared to allogeneic responses (37,161,194,265). Reactivity could often only be demonstrated by tumor cytostasis assays (15). Alternatively, *in vitro* culture with cross-reactive allogeneic tumors has been required to generate CL cross-reactive to syngeneic tumors (99,144). CL against syngeneic tumors could also be developed by culture with enzymatically modified syngeneic tumors (162) or chemically modified syngeneic tumors (78,162). These CL were much less effective than those generated from tumor-bearing mice by culture with IL2 and tumor.

3.3.9.4 COMPARISON TO GENERATION OF CL FROM TUMOR-BEARING MICE

CL activity generated by culture of spleen cells from tumor-bearing mice with IL2 and P815 cells was 100 to 1000 times higher than that reported previously for cultures

without IL2. Previously, long assay times have been necessary to demonstrate the low level of cytotoxic activity generated from tumor-bearing mice (64,95,308). Tumor cytostasis was necessary to demonstrate activity not evidenced by cytolytic activity (95,171,278). Cytolytic or cytostatic activity could be elicited in mice with small tumors but not in mice with large tumors (171,278,308).

3.3.9.5 COMPARISON TO GENERATION OF CL FROM CANCER PATIENTS

Some tumor-bearing humans have cells directly cytotoxic *in vitro* to autologous tumors (69,362). Growth on sensitizing tumor monolayers can increase this activity (120,185).

In vitro culture of cells from tumor-bearing mice and tumor-bearing humans on pooled allogeneic cells generates cytolytic activity which will lyse autologous tumors (10,185,231,323,363). Cells activated in this way, in addition to reacting with allogeneic cells, kill autologous tumor cells but not autologous normal cells. In the mouse, these culture techniques generate both CL and NK cells (231). Whether these pooled cultures are providing alloantigens cross-reactive with syngeneic tumor antigens, or providing IL2 or other lymphokines or a combination of the two is not yet clear (10,99,283).

The level of cytolytic activity generated from tumor-bearing patients was not as high as that generated in the mouse against the methylcholanthrene induced tumors, P815, L1210 and RI, but was comparable to that generated against the spontaneous murine tumor, CaD2. The level of activity generated against the autologous tumor was comparable to the level of activity of cells from allogeneic individuals against the same tumor.

3.4 SUMMARY

Cytolytic activity, specific to P815 cells, L1210 cells, RI cells, CaD2 cells, and autologous ovarian cancer cells, was developed by culture of cells from normal or tumor-bearing mice or humans with IL2 and irradiated tumor. An additional, less specific, anti-tumor activity was generated by culture with IL2 alone. Culture with IL2 and tumor is an effective method for generating cytolytic lymphocytes from tumor-bearing mice. The activity generated is equivalent to that generated against allogeneic tumors. It is higher than that previously reported for normal or tumor-bearing mice. These cells may,

therefore, be effective in immunotherapy of tumors.

4. MECHANISM OF ACTION OF IL2-GENERATED CL *IN VIVO*

4.1 INTRODUCTION

If CL-containing populations are to provide the basis for an effective immunotherapy regimen, their mode of action must be established. This will allow effective combination with other therapeutic modalities. Systems for measuring tumor clearance were developed. Utilizing these systems, both host responses and responses induced by therapy with CL-containing populations were studied.

4.2 RESULTS

4.2.1 CORRELATION OF ^{131}I-IUDR CLEARANCE WITH CELL DEATH *IN VITRO*

P815 cells lost the ability to exclude eosin (an indication of viability) within an hour following ultaviolet irradiation. When they were labeled with ^{131}I and injected into normal mice, 85% of the radio-label was cleared from mice within the first 24 hours (Figure 5). During the first two days, the rate of ^{131}I clearance from gamma-irradiated P815 cells does not significantly differ from that from normal cells. Thereafter, the clearance of ^{131}I parallels the loss of viability *in vitro* (Figure 5). Therefore, the clearance of radioactivity from mice correlates with *in vitro* cell death.

4.2.2 CL GENERATED BY CULTURE WITH IL2 ARE EFFECTIVE *IN VIVO*

Spleen cells from tumor-bearing mice, incubated with IL2 and P815 cells for 5 days, were active against syngeneic tumor in cytotoxicity assays *in vitro* (Table 5). The injection of 3.3×10^7 of these cultured spleen cells resulted in the clearance of over 90% of the radioactivity contained in 1×10^6 ^{131}I-IUdR-labeled P815 cells (injected two hours earlier) (Figure 6). Since the rate of elimination of the ^{131}I was as rapid as that from UV-irradiated P815 cells (Figure 5), the injected CL rapidly killed most of the P815 tumor cells.

If P815 cells were allowed to grow i.p. for two days before the injection of CL, the tumor was still rapidly cleared (Figure 6). Since the doubling time of P815 is short, the tumor load two days after injection of P815 cells was significantly increased. From the

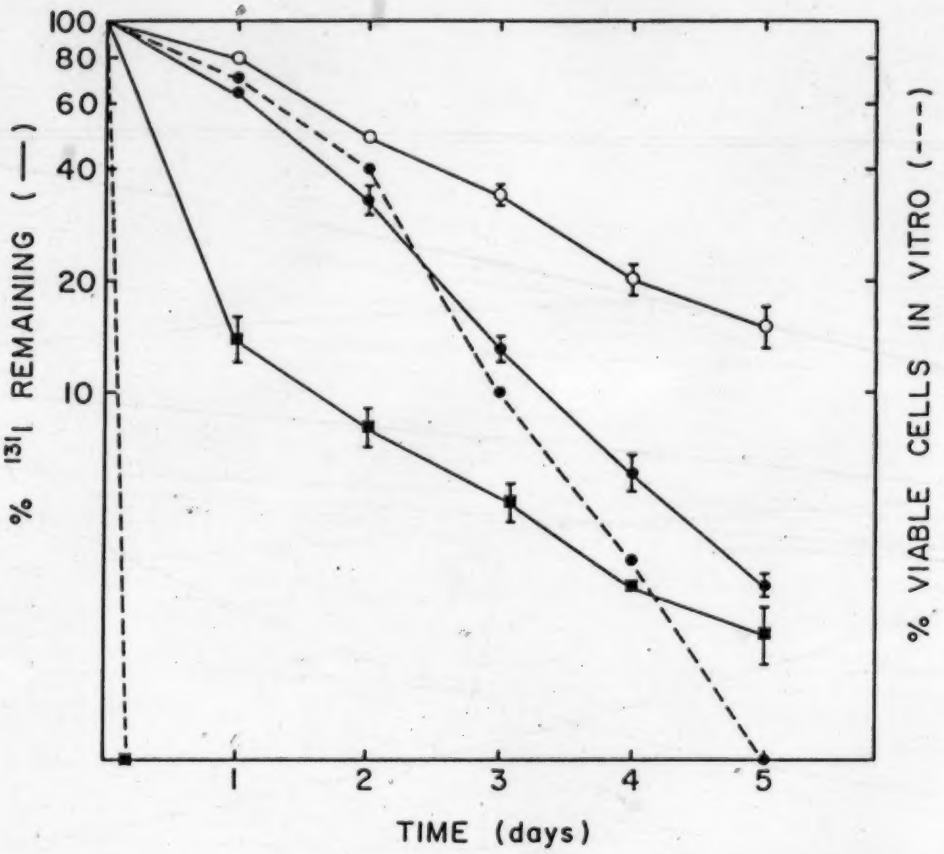

Figure 5: Survival of normal and UV or gamma-irradiated P815 cells.

Normal DBA mice were injected i.p. with 1×10^6 ^{131}I-IUdR-labeled P815 cells which were either untreated (0), gamma-irradiated (2500 rads) (0), or UV-irradiated (■). Results are expressed as the percent of the injected ^{131}I-IUdR label remaining in the mice on each day. Each group in this and all following experiments, unless otherwise stated, was composed of 5 mice.

Cells from each set were cultured at 1×10^5 cells/ml and viability checked at 2 hours and daily by eosin exclusion. Gamma irradiation (0-----0)and UV irradiation (■-------■) was as described in the Methods.

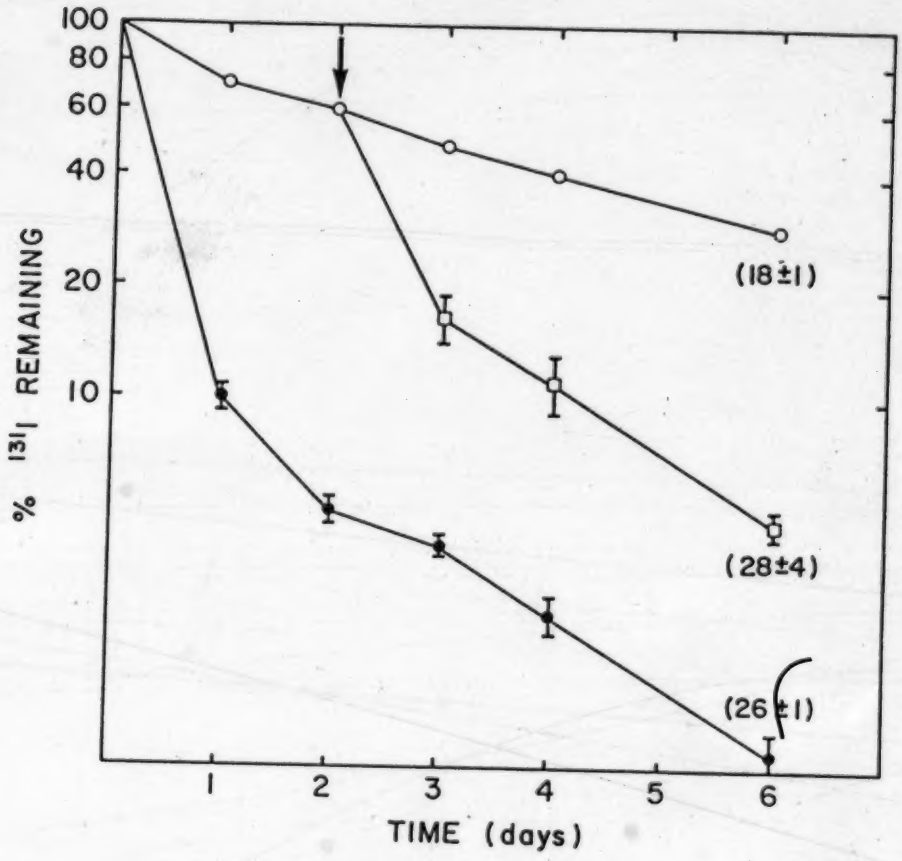

Figure 6: CL generated *in vitro* are effective *in vivo.*

Normal mice were injected i.p. with 1×10^6 ^{131}I-IUdR-labeled P815 cells on day 0 (0). Two hours later, 3×10^7 CL ($KA = 160 \times 10^{-9}$) were injected i.p.(●). These CL were generated by culturing spleen cells from mice which had been injected with 1×10^4 P815 cells 19 days earlier. The spleen cells were cultured for 5 days with P815 cells and IL2. An additional set of mice were injected i.p. with 3×10^7 CL after the initial tumor injection (arrow) (□). Mean survival times, in days, are given in parentheses.

rate of ^{131}I clearance, it appears that the CL still killed the majority of the P815 cells.

The rate of ^{131}I clearance correlated with survival times of the mice (26±1 days for treated mice versus 18±1 days for control mice, Figure 6). The mice which received CL two days after the tumor also survived longer than untreated mice (28±4 days vs 18 ±1 days).

Intraperitoneal or intravenous (i.v.) injection of CL 48 hours prior to tumor injection did not increase the rate of tumor clearance (data not shown). Also, i.v. injection of CL, two hours after the tumor, did not increase the rate of i.p. tumor clearance (data not shown). The survival times of mice receiving either of these treatments was not significantly changed by the treatment.

4.2.3 OPTIMAL CONDITIONS FOR THE GENERATION OF TUMOR-SPECIFIC CL ACTIVE *IN VIVO*

Culture of spleen cells from tumor-bearing mice with both IL2 and P815 cells generated the most effective CL. These culture conditions also resulted in the most active cells in tumor clearance (Figure 7). Spleen cells incubated with IL2 but without P815 cells slightly increased tumor clearance. Only spleen cells which had been incubated with both IL2 and P815 cells significantly improved survival times. Two of the five treated mice survived for more than one year despite having received at least 2×10^4 times the lethal i.p. tumor dose. The tumor had been growing i.p for two days prior to CL treatment. We have never observed survivors in untreated mice given this tumor dose.

Spleen cells from normal mice cultured with IL2 and P815 cells were cytotoxic to P815 cells *in vivo* (Figure 8). These cells were not as active as spleen cells from tumor-bearing mice but they still significantly increased the rate of tumor clearance. These CL did not significantly improve survival times for those mice dying of the tumor (26±7 days for treated mice vs 20±2 days for control mice).

Injection of large numbers (1×10^8) of spleen cells directly from normal or tumor-bearing mice did not increase either tumor clearance or survival times. Culture with IL2 and P815 cells was necessary to generate effective CL *in vitro* and also to generate cells active *in vivo* in tumor clearance and in improving survival times.

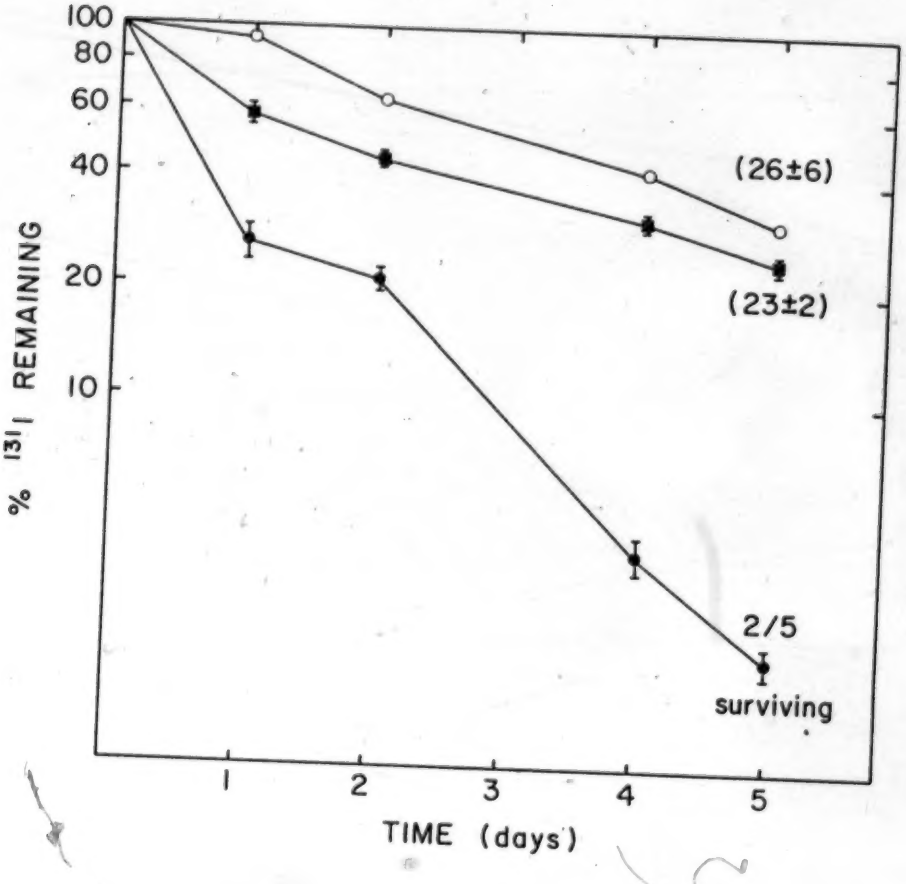

Figure 7: Conditions for the *in vitro* generation of CL from tumor-bearing mice
Normal DBA mice were injected i.p. with $1x10^6$ ^{131}I-IUdR-labeled P815 cells two days prior to CL injection (i.e. day -2) (0). On day 0, mice were injected i.p. with $1x10^7$ spleen cells from 21-day tumor-bearing mice which had been cultured alone (KA<$5x10^{-9}$), with P815 cells (KA<$5x10^{-9}$, with IL2 alone (KA=$200x10^{-9}$) (■), and with P815 cells and IL2 (KA=$700x10^{-9}$) (●). The clearance of ^{131}I-IUdR from mice which were injected with spleen cells cultured alone or with P815 cells alone were superimposible on the curve for untreated mice and are therefore not indicated separately. The number of counts remaining in the mice on day 0 was arbitrarily assigned a value of 100%. By day 2 (4 days after the original tumor injection), the counts remaining in the mice were low requiring an additional injection of ^{131}I-IUdR-labeled P815 to serve as a marker of survival of the tumor. This did not significantly change the survival time of a separate group of mice. Survival times of groups in which there were no "cures" and the number of mice surviving indefinitely from the CL treated group, are included in the figure. Untreated mice survived 26±6 days while mice treated with cells cultured alone or with P815 cells survived 21±0 and 25±2 days, respectively.

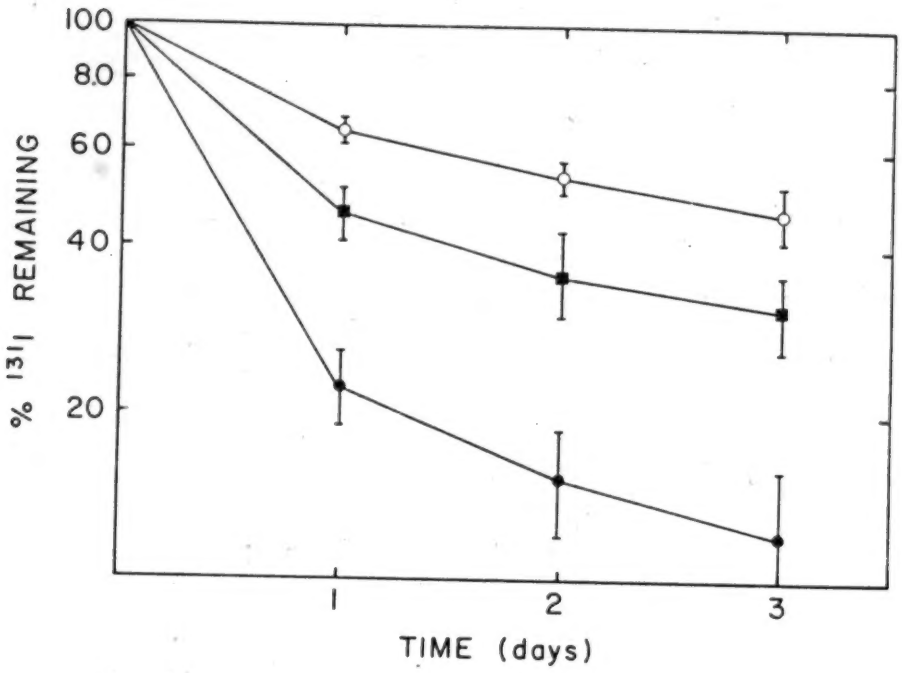

Figure 8: Optimal conditions for *in vitro* generation of CL from normal mice

CL, 1×10^7, generated from spleen cells from normal mice were injected i.p. two hours after 1×10^6 ^{131}I-IUdR-labeled P815 cells. Spleen cells were either cultured alone, with IL2 (KA=10×10^{-3} (■), or with IL2 and P815 cells (KA=40×10^{-3})(●). Control mice, mice treated with spleen cells cultured with medium alone (KA<5×10^{-3}) or with P815 cells (KA<5×10^{-3}) all gave superimposible curves (○). Survival times of the mice were not significantly changed by any of the treatments except that one mouse treated with CL generated by culture with IL2 alone survived for more than one year.

4.2.4 TUMOR CLEARANCE CORRELATES WITH NUMBER OF CL INJECTED

Initial clearance of ^{131}I-IUdR correlates with the number of injected CL (Figure 9). Over the range of CL injected, 3-30x10^6, there was no change in improvement of survival.

4.2.5 HOST RESPONSE TO P815

Mice which had been injected with tumor 7 days prior to injection of ^{131}I-IUdR-labeled P815 cells cleared tumor more rapidly than mice given tumor for the first time (Figure 10). This increased rate of tumor clearance was abrogated by irradiation (600 rads) of the mice (Figure 10). DBA mice demonstrated a transient, radio-sensitive cytolytic response against P815.

4.2.6 SPLEEN CELLS CULTURED WITH IL2 AND P815 CELLS IMPROVE THE HOST RESPONSE TO P815

Injection of CL-containing populations on day 0 augmented the natural host response against P815 cells on day 7 (Figure 10). This increased response was further augmented by irradiation of the mice. This suggests that the host response augmented by the injected CL population is modulated by a radio-sensitive host suppressor cell.

The cytotoxic response assayed on day 7 correlated with increased survival times. Treated irradiated mice survived almost three times as long as irradiated control mice (36 days vs 13 days) and twice as long as non-irradiated control mice (36 days vs 18 days).

4.2.7 INDUCED ANTI-TUMOR ACTIVITY CORRELATES WITH NUMBER OF CL INJECTED *IN VIVO*

The induced response *in vivo* (as reflected by clearance of tumor cells 7 days later) correlates with the number of CL initially injected (Figure 11). As stated previously, survival times also correlated with numbers of CL injected.

4.2.8 ORIGIN OF THE AUGMENTED HOST RESPONSE

The improved host response may have been due to a reduction in tumor burden by the administered CL. To test this, UV-irradiated or gamma-irradiated P815 cells were injected into DBA mice. These tumor cells were incapable of growing and increasing the

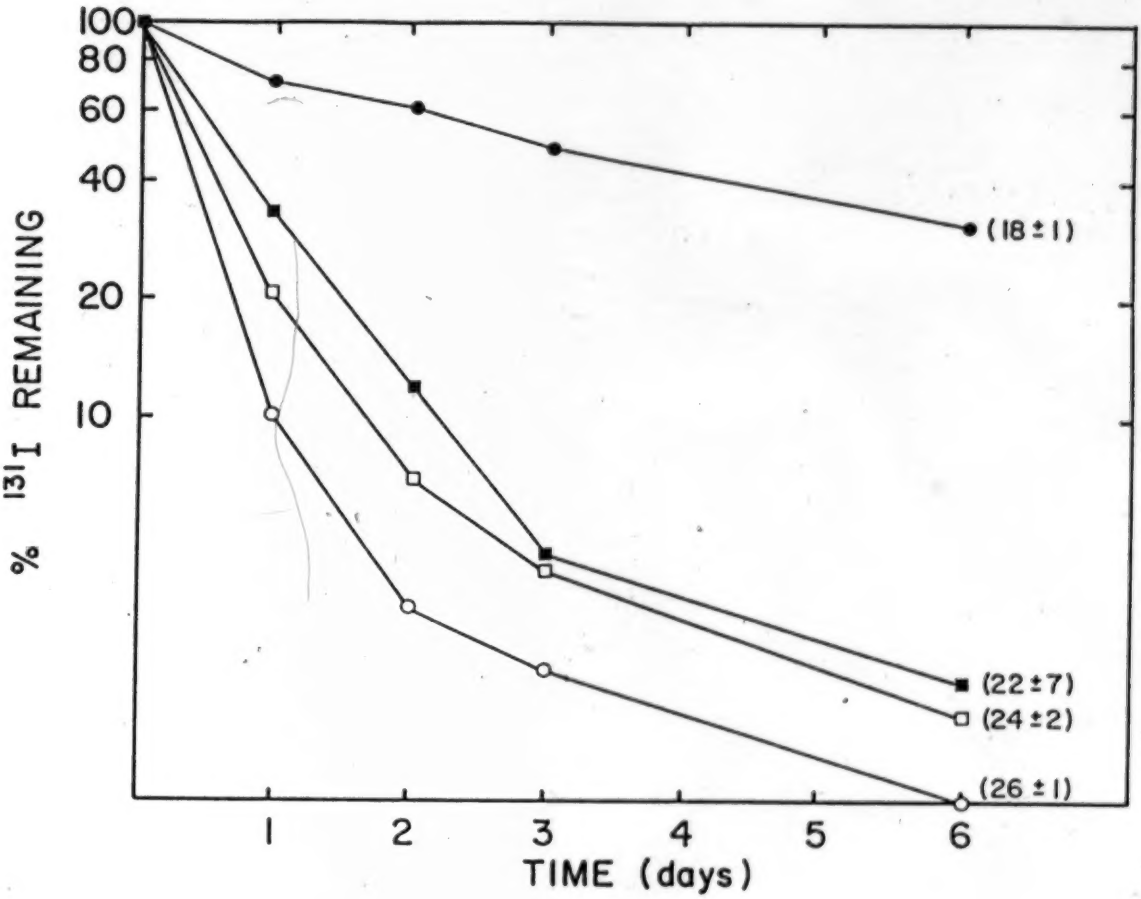

Figure 9: Dilution curve of *in vitro* generated CL *in vivo*

As part of the experiment presented in Figure 7, a dilution curve of CL activity was performed. Spleen cells cultured with IL2 and P815 cells, 3×10^6 (●), 1×10^7 (■), and 3×10^7 (0) were injected i.p. All mice received 1×10^6 ^{131}I-IUdR-labeled P815 (●) prior to injection of CL.

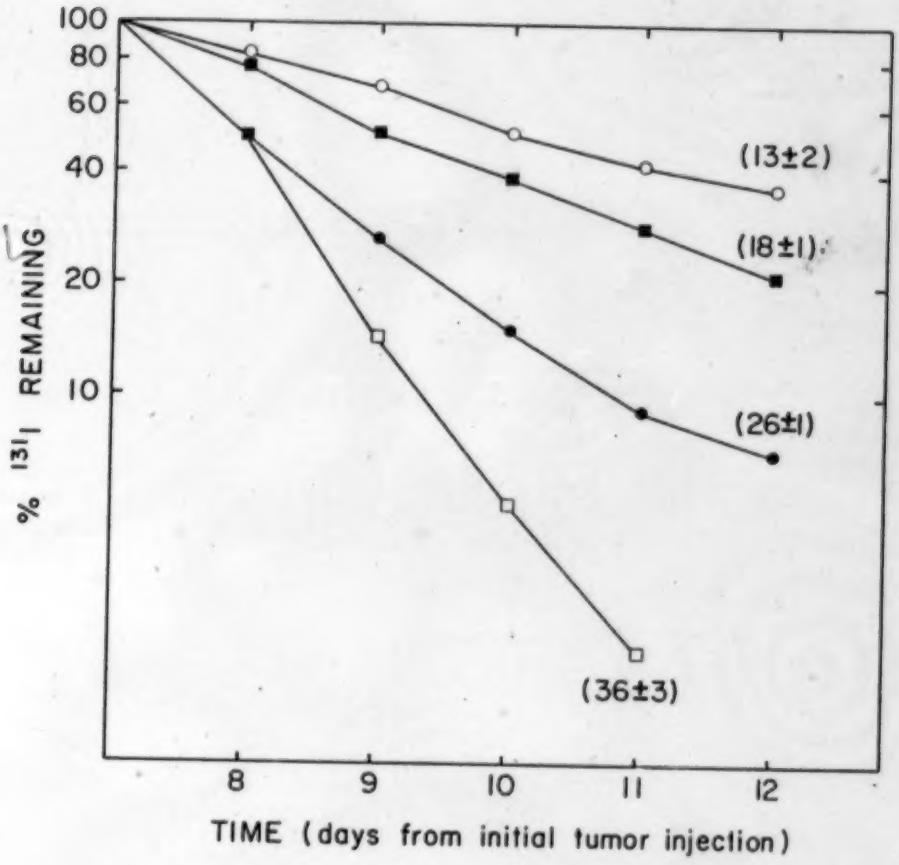

Figure 10: Secondary tumor clearance.

Mice which had been injected with 1×10^6 ^{131}I-IUdR-labeled P815 cells were injected with an additional 1×10^6 ^{131}I-IUdR-labeled P815 cells 7 days later (■). In one group(●), CL, 3×10^7 (KA=160×10^{-9}), were injected 2 hours after the initial injection of P815 cells. In addition, two sets of mice were irradiated with 600 rads of gamma-irradiation two days prior to the initial P815 injection (i.e. day -2). These mice then received either P815 cells alone (0) or P815 cells and CL (□). The initial rate of ^{131}I-IUdR clearance for the irradiated mice was indistinguishable from non-irradiated mice given in figure 7. Control mice, injected with P815 cells on day 7 only, cleared ^{131}I-IUdR indistinguishably from the irradiated mice. Survival times, in days, are indicated for the given curves.

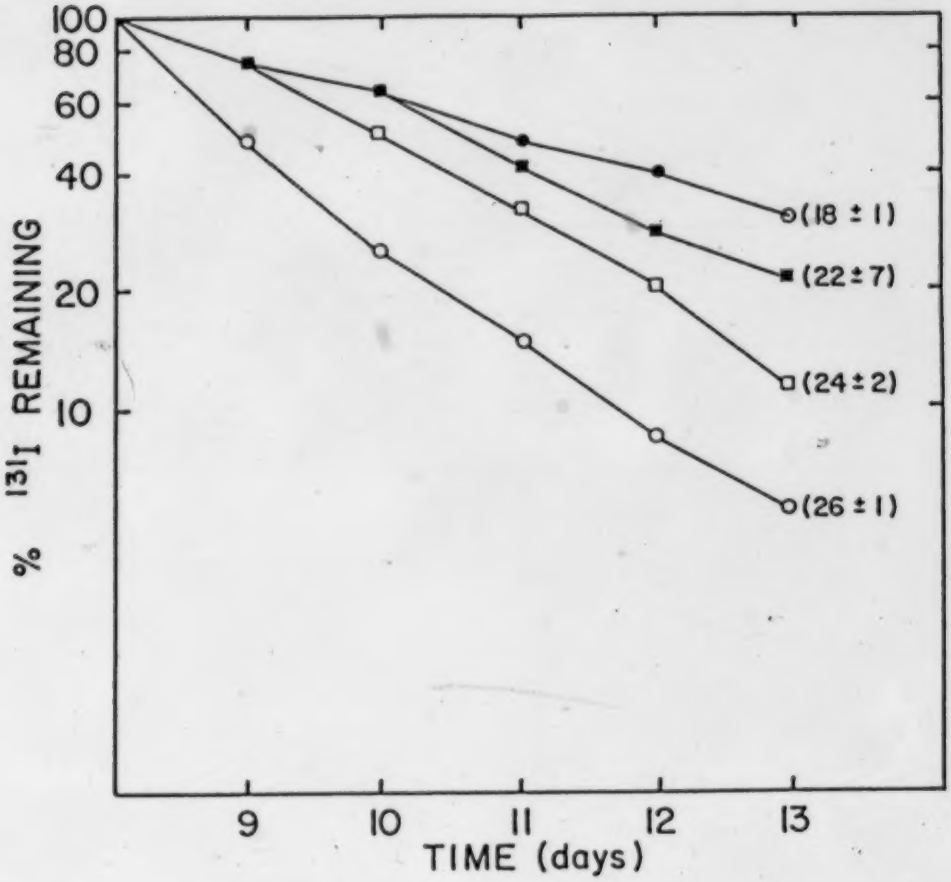

Figure 11: Dilution curve of induced responses.

Induced response demonstrates a dilution curve correlating with initial tumor clearance. The delayed response of mice initially injected i.p. with radio-labeled P815 cells (•) and $3x10^6$ CL (KA=$160x10^{-8}$) (■), $1x10^7$ CL (□) or $3x10^7$ CL (o), was assessed by injecting $1x10^6$ radio-labeled P815 8 days later. The initial clearance of ^{131}I-IUdR from these mice is given in Figure 9.

tumor burden. The rate of clearance of tumor injected 7 days later was similar to that of mice which received untreated tumor (Figure 12). Injection of UV killed or radiation-blocked tumor did not result in an improved host response, suggesting that the injected CL population played a direct role in potentiating the host response (Figure 10).

4.2.9 SURVIVAL OF INJECTED CL

The initially injected CL may have persisted *in vivo* long enough to kill the tumor injected on day 7. Therefore, ^{125}I-IUdR labeled CL were injected into DBA mice (Figure 13). The initial rate of clearance of label by these mice was similar to that of mice injected with UV-irradiated P815 cells (Figure 5). Over 80% and 85% of label from CL injected i.p. or i.v., respectively, was cleared in the first 24 hours following injection (Figure 13). The rate of clearance was not affected by the injection of 1×10^6 P815 cells i.p. (data not shown). Therefore, the majority of the injected CL did not survive long enough *in vivo* to mediate the delayed tumor clearance. This does not, however, rule out the possibility that a small portion of the injected cells survive and perhaps even proliferate *in vivo*.

4.2.10 REQUIREMENT FOR CL TO PERSIST OR PROLIFERATE *IN VIVO*

Although initial tumor clearance was not decreased by irradiation of the CL (data not presented), irradiated CL were not capable of inducing a significant delayed host response (Figure 14). The induced host activity was decreased to levels similar to that of untreated mice. These mice exhibited slightly improved survival times (25±3 vs 18± 1).

Although the majority of the injected CL disappeared rapidly *in vivo* (Figure 13), irradiation interfered with the cell augmenting the delayed host response. Whether this is by preventing proliferation of the injected CL or some other mechanism is unknown. Improved survival times appeared to be due to both the initial tumor clearance and the induced response since survival times of mice treated with irradiated CL were intermediate between non-treated mice and mice treated with non-irradiated CL (Figure 13).

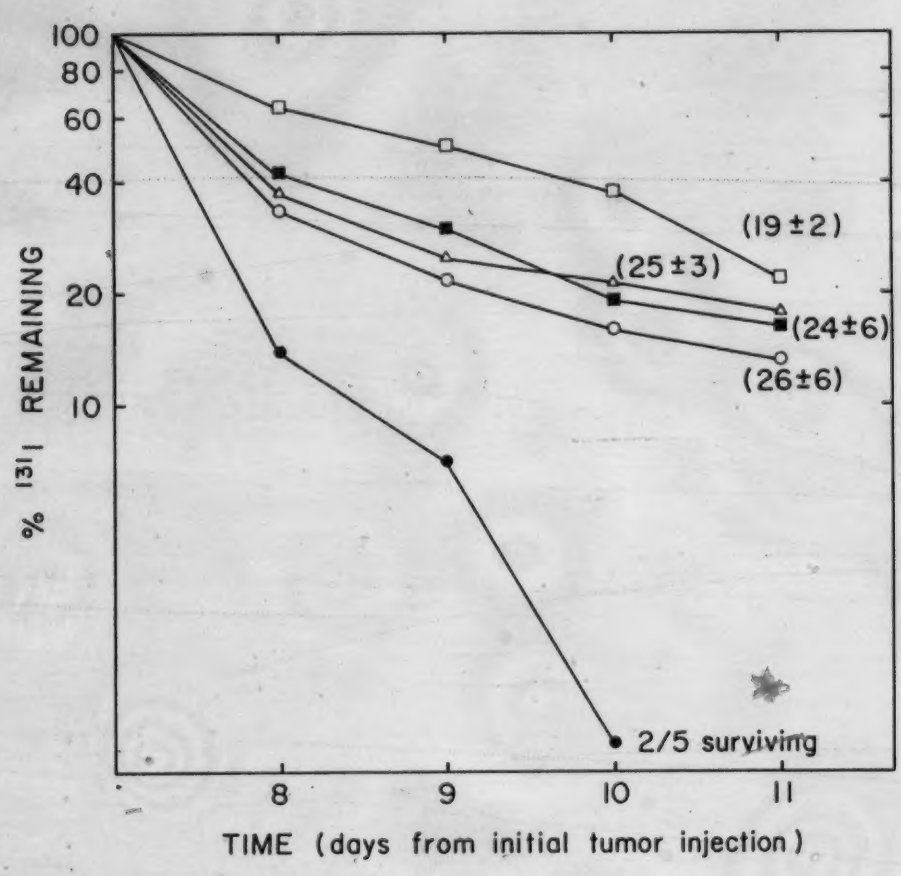

Figure 12: The improved host response is not due to decreased tumor burden.

The delayed response of mice initially injected with ^{131}I-IUdR-labeled P815 cells (0), UV-irradiated P815 cells (▲), gamma-irradiated P815 cells (■) or ^{131}I-IUdR-labeled P815 cells plus 1×10^7 CL (KA=700×10^{-4}) (●) was assessed by injecting 1×10^6 ^{131}I-IUdR-labeled P815 cells 7 days after the initial tumor cell injections. Where indicated, the CL were injected 2 days after the initial injection of P815 cells. Control mice (0) were injected with ^{131}I-IUdR-labeled cells on day 7 only. The initial ^{131}I-IUdR clearance for these mice is given in Figures 5 and 7.

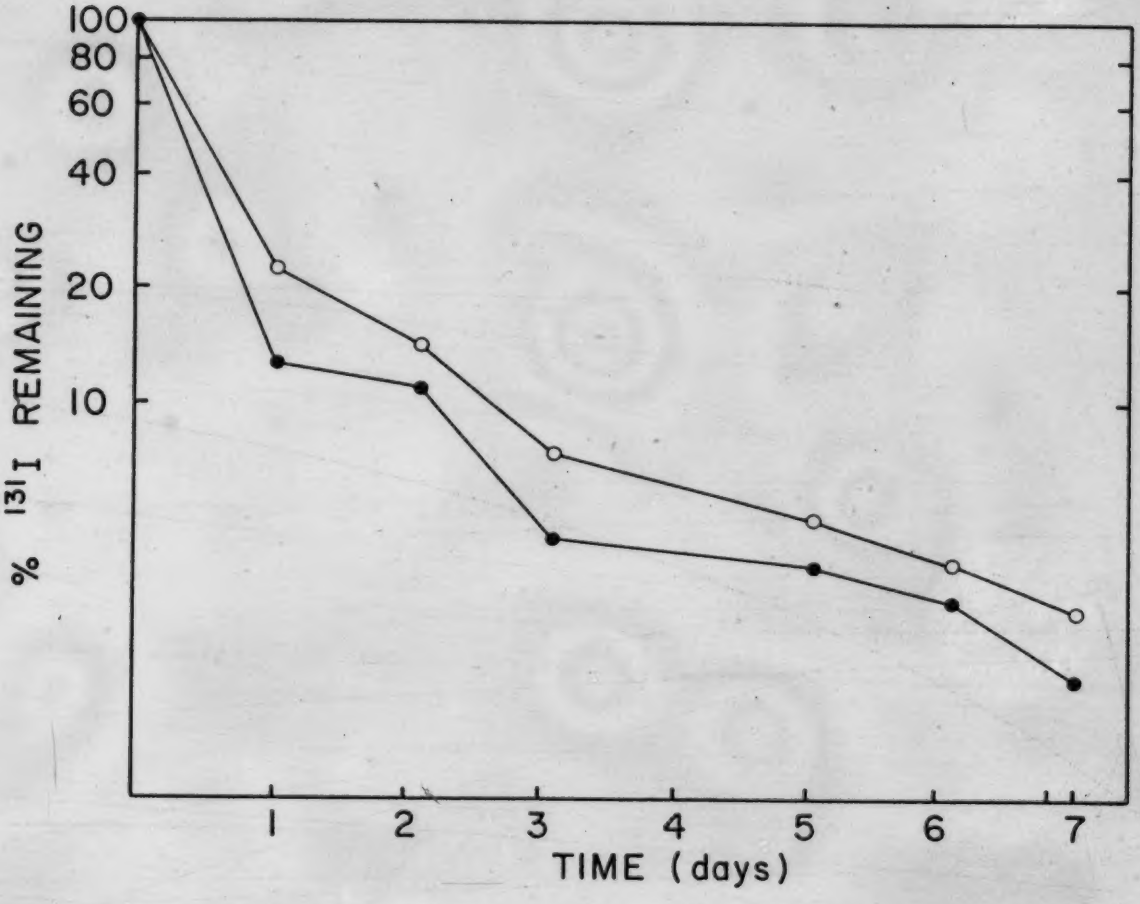

Figure 13: Survival of CL *in vivo.*

Spleen cells from tumor-bearing mice were cultured for 5 days with IL2 and P815 cells. For the final 18 hours of culture, 0.5 microcuries of ^{131}I-IUdR were added. Cultured spleen cells, 1×10^7, were injected i.p. (0) or s.c. (●) into normal mice. Injection of P815 cells i.p. 2 hours prior to CL did not change the ^{131}I-IUdR clearance rate.

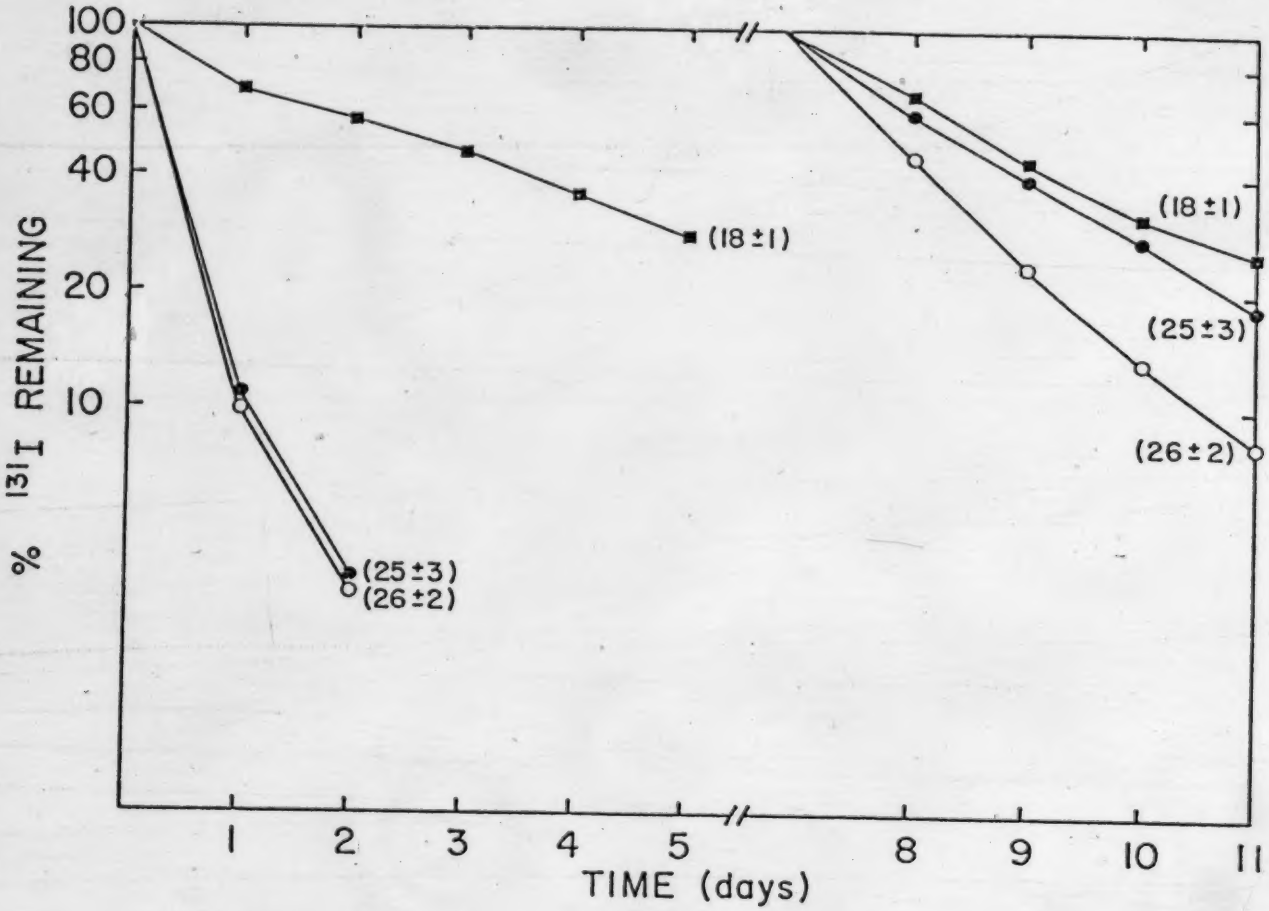

Figure 14: Efficacy of irradiated CL *in vivo*.

CL generated from tumor-bearing mice by culture with P815 cells and IL2 were irradiated with 2500 rads. Irradiated CL, 3×10^7, were injected i.p. (●) 2 hours after 1×10^6 ^{131}I-IUdR-labeled P815 cells. Control mice received 3×10^7 non-irradiated CL (○) or no treatement (■). Control mice are the same as in Figures 6, 9, and 11. All mice received an additional 1×10^6 ^{131}I-IUdR-labeled P815 cells i.p. on day 7 in order to follow delayed responses.

4.2.11 CL ARE ALSO ACTIVE AGAINST L1210 *IN VIVO*

CL were generated from spleen cells from L1210-bearing mice by culture with IL2 and L1210 cells. Although *in vitro* activity was low (KA of 38×10^{-8}), the cells still mediated tumor clearance, induced a host response and perhaps slightly improved survival times of some mice (19±7 days, treated vs 14±1 days, control) (Figure 15).

4.2.12 SPECIFICITY OF SYNGENEIC CL IN VIVO

CL generated by incubating spleen cells with IL2 and syngeneic tumor were able to kill at least two different syngeneic tumor targets *in vivo* (Table 27). Spleen cells from mice with P815 tumors, following culture with IL2 and P815 cells, killed both P815 cells and L1210 cells *in vivo*. Spleen cells from mice with L1210 tumors, following culture with IL2 and L1210 cells, killed both P815 cells and L1210 cells *in vivo*. Therefore, CL generated from mice with either P815 or L1210 tumors improved the host response to both tumors. As only 1×10^6 CL were injected to allow assessment of cross-reactivity, the CL were insufficient to improve survival times (data not shown).

4.2.13 CL ACTIVITY IS NOT BLOCKED BY SUPPRESSOR ACTIVITY IN TUMOR-BEARING MICE

CL generated *in vitro* were as effective in tumor-bearing mice as they were in normal mice. Subcutaneous P815 tumors were surgically resected to allow the mice to fit into the counting chambers. Surgical resection of the tumor did not improve survival times. Therefore, the mice must have had extensive metastatic tumor. Since these mice had had tumor for 20 days, any possible *in vivo* suppressor effects, induced by the tumor, were expected to be active. When an additional 1×10^6 radio-labeled P815 cells were injected, 12±3% of the ^{131}I was cleared from the surgically resected tumor-bearing mice within the first 24 hours. Within the same time period, 25±5% of the ^{131}I was cleared from normal mice. CL, 1×10^6, injected i.p. cleared an additional 28% of the tumor from both tumor-bearing and normal mice. CL generated *in vitro*, therefore, were effective *in vivo* in tumor-bearing mice.

Without additional treatment the tumor-bearing mice cleared less tumor than did normal mice (12±3% vs 25±5%). This suggests that mice have a mechanism for the early

Table 27

Specificity of CL *in vivo*

CL generated from mice with	Response on day 1 % Targets killed Assay tumor P815	L1210
P815	37	20
L1210	63	44

	Response on day 7 P815/P815	P815/L1210	L1210/L1210	L1210/P815
P815	16	16	30	27
L1210	23	ND	27	ND

CL were generated from P815 and L1210 tumor-bearing mice injected s.c. 15 days previously (1×10^3 and 1×10^4, respectively). These CL were assayed by injecting a low number, 1×10^6, into mice two days after the test tumors, as indicated The numbers of CL injected were kept low to allow comparison of the cross-reactivity. The first part of the table gives the initial increase in clearance of ^{131}I-IUdR from treated mice compared to control mice 24 hours after the initial injections of CL, (CL treated clearance-Control clearance)/Control clearance The second part of the table indicates the clearance of tumor in the 24 hours following a second radio-labeled tumor injection 7 days after the first injection, without any additional CL. In the second part of the table, the designation across the top represents the tumor injected on day 0 followed by the tumor injected on day 7. Survival times of all of the treated and untreated normal mice were 14 ± 9 for the mice that received L1210 cells first and 22± for the mice that received P815 cells first. The second tumor injected did not affect the survival time.

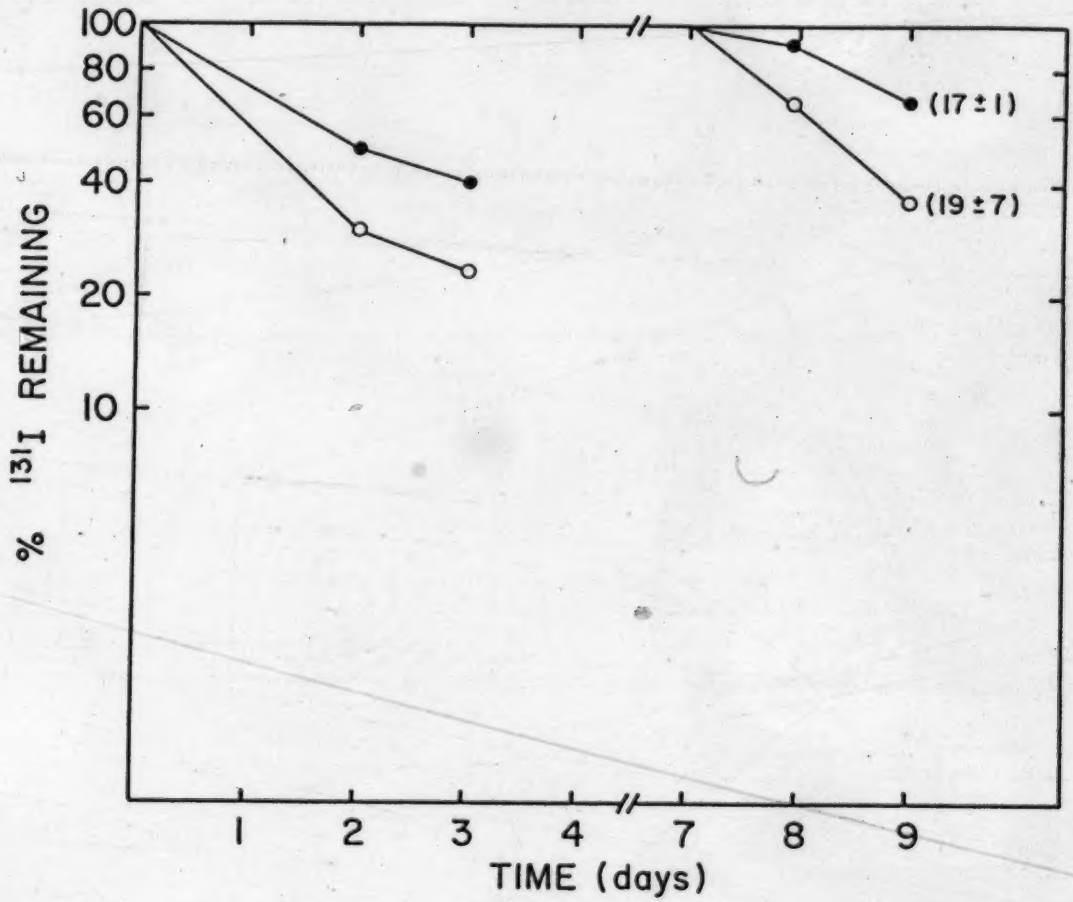

Figure 15: Activity of anti-L1210 CL *in vivo*.

Spleen cells from L1210 tumor-bearing mice were cultured with L1210 cells and IL2. Cultured cells, 1×10^7, were injected 2 days following the i.p. injection of 1×10^6 ^{131}I-IUdR-labeled L1210 cells (○). Control mice received tumor only (●). An additional 1×10^6 ^{131}I-IUdR-labeled L1210 cells were injected on day 7 to follow delayed responses.

clearance of tumor cells. This mechanism is not sufficient to prevent the overgrowth of P815.

4.2.14 CL IMPROVE SURVIVAL TIMES OF MICE WITH I.P. OR S.C. TUMORS

Treatment with CL improved the survival times of mice which received 1×10^3 P815 cells i.p. (Table 28). CL given i.v. were effective in improving survival times only when CL were also injected directly into the primary tumor mass. Even then, CL were only effective i.v. when the tumor had been present for at least two days prior to CL injection.

Treatment of mice with s.c. tumors showed a slightly different pattern of response than mice with i.p. tumors. Three out of five mice with s.c. tumors treated with both s.c. and i.v. CL survived the tumors (Table 28). The survival times of the treated mice that died of the tumor were the same as those of untreated mice. In other words, in some mice the CL cured the s.c. tumor, while for others the CL therapy was ineffectual.

4.2.15 CL GIVEN I.V. HOME TO S.C. TUMOR

CL i.v. improved survival times of surgically treated tumor-bearing mice and of mice with s.c. tumors (Table 28). ^{131}I-IUdR-labeled CL were, therefore, injected i.v. into tumor-bearing mice to assess their ability to localize to tumor (Table 29). The majority of the CL localized to the lung. Mice bearing the specific sensitizing tumor demonstrated significant concentrations of ^{131}I-IUdR and therefore CL in the tumor mass. CL did not localize to the same area in normal mice, in mice with recent tumor injections or in mice with large CaD2 or L1210 tumors.

The CL that localized to the P815 tumor comprised 10% of the total counts of ^{131}I-IUdR in the mice. The P815 tumors averaged 10 gm wet weight with 150 counts / min / gm wet weight. The CaD2 tumors had only 50 counts / min / gm wet weight which comprised only 5% of the total counts in the mice. The L1210 tumor had 25 counts / min / gm wet weight comprising 3% of the total counts in the mice. The CaD2 and L1210 tumors were all between 8 and 12 gm. This experiment was performed only once and the results must be considered as preliminary.

Table 28

Survival of Mice With Small Tumors

Treatment	Survival Time	Long Term Survivors
I. Subcutaneous tumor		
None	29±4	0/26
CL s.c.	30±5	1/5
CL s.c. and i.v.	26±5	3/5
Overall CL Treated	28±7	4/10
II. Intraperitoneal Tumor		
None	24±2	0/14
CL i.p. same day	39±7	1/5
CL i.p. and i.v. same day	34±4	0/5
CL i.p. 3 days following tumor	28±2	0/4
CL i.p. and i.v. 3 days following tumor	36±6	0/5
Overall CL Treated	36±9	1/19
III. Overall Results		
Untreated		0/40
Treated		5/29

DBA mice were injected with 1×10^4 P815 cells either i.p. or s.c. as indicated. CL, 1×10^7, were injected as listed. Survival times are in days following initial tumor injection for mice which died of the tumor.

Table 29

Homing of CL to Tumor P815

Location	Tumor type in Mouse Relative radioactivity			
	none	P815	CaD_2	L1210
Tumor Mass	4	54	< 5	7
Spleen	7	10	4	< 5
Liver	37	37	12	20
Kidneys	13	21	< 5	7

Spleen cells from P815 tumor-bearing mice were incubated with P815 cells and IL2. These cells were incubated with ^{125}I-IUdR for the final 18 hours of culture. CL, 1.5×10^7, were injected i.v. Tumor-bearing mice had been injected with 1×10^4 P815 cells, 1×10^4 CaD2 cells, or 1×10^3 L1210 cells 21 days prior to use. The indicated organs were excised and counted 18 hours following i.v. injection of CL. There were 4 mice in each group. Data are expressed relative to the radioactivity retained in the lungs (ca. 10-15% of the total injected radioactivity). Values are expressed as (counts/min in organ/counts/min in lung) x 100. All tumor masses were between 8 and 12 grams.

4.2.16 INTRAVENOUS CL CAN LOCALIZE TO INTRAVENOUS TUMORS

Because a significant amount of tumor spread is via the blood stream, attempts were made to clear i.v. injected tumor with i.v. CL. Following i.v. injection of ^{131}I-IUdR-labeled P815 cells the radio-label rapidly disappeared (data not shown). This made ^{131}I-IUdR clearance assays impossible. The reason for this loss of radio-label is unknown.

Intravenous injections of CL were able to improve survival times of some mice injected i.v. with tumor (Figure 16). Therefore, CL were able to travel to at least some of the same sites as did the initial i.v. tumor injection. Intravenous injections of P815 cells resulted in death in 30±4 days. Intravenous CL therapy prolonging life to 44±13 days. The CL therapy had little effect on the survival of the two mice that died early, but did significantly improve the survival time of the other three mice.

4.2.17 CL TREATED MICE HAVE LONG-TERM RESISTANCE TO TUMOR CHALLENGE

Mice which had survived injection of P815 cells because of treatment with CL were resistant to further challenge with P815 cells. All of these mice (10 / 10) survived repeated challenges with 1×10^6 P815 cells s.c. or i.p. up to one year after the initial CL treatment. Untreated mice did not survive the same tumor dose (0 / 20).

4.3 DISCUSSION

4.3.1 INITIAL TUMOR CLEARANCE

Tumor cell killing by CL *in vivo* was followed by monitoring retention of ^{131}I-IUdR label from ^{131}I-IUdR labeled tumor cells injected i.p. Accelerated tumor clearance correlated with the *in vitro* activity of the CL. Only cells demonstrating significant *in vitro* cytolytic activity (i.e. cells from tumor-bearing mice cultured with IL2 and P815 cells, Figure 7) mediated *in vivo* tumor clearance and improved survival times. CL generated from either L1210 or P815 tumor-bearing mice (Figure 6, 15) and possibly normal DBA mice (Figure 8) were effective in clearing the sensitizing tumor *in vivo*. A considerable degree of cross-reactivity was observed between CL generated against P815 cells and L1210 cells both *in vitro* and *in vivo* (Table 9,27).

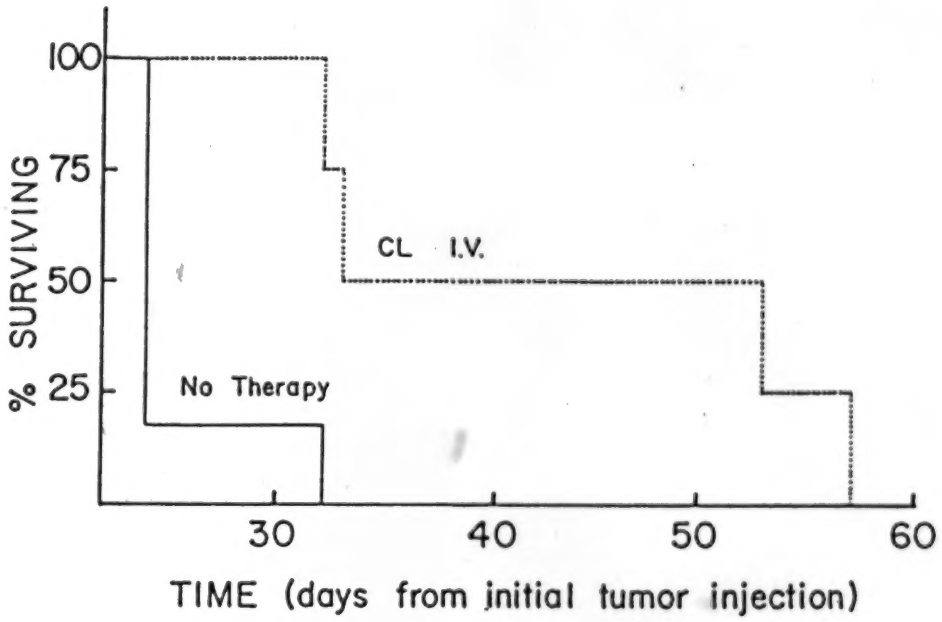

Figure 16: CL i.v. improve survival times of mice given i.v. tumor.

Nine mice were injected with 1×10^6 ^{131}I-IUdR-labeled P815 cells i.v. Four of these mice received 1×10^7 CL i.v. 2 hours later. The mean survival time of the untreated mice (——) was 30±4 days while CL treated mice (-------) survived 44±13 days.

To be effective in initial tumor clearance, CL-containing populations needed to be injected directly into the tumor site. They could be irradiated. They were effective in tumor-bearing or irradiated mice. The CL did not need to survive for long periods of time *in vivo*. The initial tumor clearance was probably directly related to the injection of CL rather than to other cell types contained in the CL preparations.

4.3.2 INITIAL HOST RESPONSE TO P815

In the first 24 hours after the injection of labeled P815 cells into normal DBA mice, an increased rate of ^{131}I clearance occured relative to the rate of clearance over the following 4-5 days (data not shown). This increased rate of tumor clearance did not occur in tumor-bearing mice, suggesting that a small early "natural" host response occured against P815 tumor in normal mice and that this response was exhausted in tumor-bearing mice. This response could be in the form of natural killers, activated macrophages, natural antibody or a combination of any of these.

4.3.3 DELAYED HOST RESPONSE TO P815

Following injection of transplantable tumor cells, normal mice often demonstrate a transient immune response 6-7 days later. This transient response often results in the temporary cessation of *in vivo* tumor growth (19,308). This response can be demonstrated, indirectly, by "concomitant" immunity. As mentioned previously, concomitant immunity is defined as the ability of a mouse with a tumor growing at one site to prevent the growth of an injection of tumor cells at another site (19,20,75). The response of mice against autologous tumor can also be demonstrated by adoptive transfer of concomitant immunity (61). The increased rate of clearance of a second injection of ^{131}I-IUdR labeled tumor cells, 7-9 days after an initial tumor injection into otherwise untreated mice (Figure 10), directly demonstrated this host cytolytic response. Irradiation of the mice removed this response (Figure 10).

4.3.4 AUGMENTATION OF DELAYED RESPONSE TO TUMOR BY CL THERAPY

CL-containing populations injected into mice with i.p. tumors not only directly mediated tumor killing but also induced a significant delayed response to the tumor (Figure 10). This increased delayed response is not simply due to prevention of tumor growth by the CL-containing populations, since injection of UV-irradiated or gamma-irradiated tumor cells did not result in a host response of similar magnitude (Figure 12). The induced response was potentiated by sublethal irradiation of the mice (Figure 10). This irradiation may be preventing the action of a host suppressor cell population (20,129,223,308,309). Despite the delayed response, the mice still did not usually survive the tumor. Those that did survive the tumor remain tumor-immune. The failure of mice to survive the tumor may be due to delayed generation of host suppressor cells (20), to inability of the CL to reach all tumor sites (37,60,190), to resistant tumor variants, or to other causes.

4.3.4.1 ROLE OF INJECTED CELLS IN DELAYED RESPONSE

Although the majority of the injected CL disappeared rapidly (Figure 13), it is not possible to rule out the survival and proliferation of a small portion of the injected lymphocytes. Irradiation of the CL did not decrease initial tumor clearance *in vivo* but did decrease the delayed tumor clearance without significantly decreasing improvement in survival times (Figure 14). Irradiation of effector cells has been demonstrated to have little effect on *in vitro* cytolytic activity (127,128). Irradiation of the same effector cells significantly decreased the ability to transfer adoptive immunity (127,128).

It is not possible to rule out the delayed response being due to proliferation of the injected lymphocytes rather than to a recruited host response. This seems unlikely both due to the *in vivo* survival of very few injected cells and due to the long period of resistance to tumor challenge that the surviving mice exhibited.

4.3.5 CHARACTERIZATION OF THE INJECTED CELL RESPONSIBLE FOR IMPROVING SURVIVAL TIMES

Recruitment of host effector cells by cultured lymphocytes (17,317,321) and by immune lymphocytes (119,129,250) has led to improved survival in adoptive immunotherapy studies. This recruitment is augmented by interference with a population of suppressor cells by adult thymectomy and irradiation (223), by irradiation (20,76,88) or

by treatment with cyclophosphamide (86,129,222,268). Therapy with CL populations was more effective in sublethally irradiated mice than in normal mice (Figure 10), probably because of interference with this suppressor population and subsequent recruitment of a host response by helper cells injected with the CL.

Unfortunately, it is not possible to separate CL from helper cells on the basis of surface markers in the DBA mouse. Separation of helper cells from CL in other murine systems has confirmed that the cell active in immunotherapy with immune spleen cells is an Ly 1^+2^- helper or DTH-mediating cell rather than an Ly 2^+3^+ CL (128,129). In rats, $W3/25^+$ helper cells rather than CL are the effector cells in adoptive immunity (88,89,90).

Injection of long term CL lines and clones has generally been ineffective in prolonging survival of tumor-bearing mice. They are effective only if injected as a tumor-CL admixture (Winn or tumor neutralization assay) (110,163,291). CL lines can be effective in prolonging survival of tumor-bearing mice, if IL2 is injected with the cultured cells (51,52,89,90,268). When a CL population which was probably free of helper cells (from the cell line developed from P815-bearing mice by culture with IL2, Table 23) was assayed, it was not effective in either initial tumor clearance or in prolonging survival of mice (data not shown). The level of cytolytic activity and cell number was low suggesting that the lack of killing may have resulted from lack of injection of sufficient CL activity.

It is attractive to hypothesize that the effective populations of injected cells contained both CL and helper cells. The CL decreased the tumor load by killing tumor cells directly. A helper cell population, which had to survive longer or proliferate, then recruited a host response. The survival of the mice depended on the balance between these two effector mechanisms and a radio-sensitive population of cells capable of suppressing the host response and possibly suppressing the injected cells directly.

4.3.6 ABILITY OF INJECTED CELLS TO HOME TO TUMOR

Although we have demonstrated that CL can be generated from tumor-bearing mice and are effective in tumor clearance and inducing host responses *in vivo*, it must be demonstrated that CL can home to tumor cells *in vivo* before they can be considered to be useful for immunotherapy in most human tumor systems (351). CL injected i.v., two days before or at the same time as injection of the tumor i.p., did not mediate initial tumor

clearance or improve survival times (data not presented). This suggests that CL do not home to fresh tumor implants. This was confirmed by injecting ^{125}I-IUdR labeled CL into mice with 5-day s.c. P815 and CaD2 tumors. Not only was there no excess radio-label in the area of the tumor compared to control mice but most of the radio-label was trapped in the lung (data not shown).

CL were able to home to large tumor implants (Table 30). A major portion of the retained radioactivity was located in the lung. The number of cells which homed to the tumor site, although significant, was small.

CL injected i.v. improved survival times of mice receiving P815 cells i.v. (Figure 17). This suggests that CL injected i.v. could travel to tumors which spread by the i.v. route. The improved survival of mice with s.c. P815 tumors treated with CL s.c. and i.v. (3/5 surviving, Table 28), of mice with i.p. P815 tumors treated with CL i.p. and i.v. (Table 28) and of mice with surgically resected CaD2 tumor treated with i.v. CL (4/4 surviving, Figure 23) provide direct evidence for the ability of injected CL to travel to metastatic tumor *in vivo*.

Although populations of CL induced in culture and long term CL lines seem to circulate abnormally *in vivo* (Table 28) (37,60,190,260), i.v. injections of cells cultured from immune animals have been effective in controlling solid tumors or metastases (21,48,49,50,88,129,322). A significant number of the infused cells must reach the appropriate sites.

4.3.7 SAFETY OF CL THERAPY

The safety of CL therapy was suggested by the failure of CL to lyse normal DBA cells *in vitro* (Table 8). In addition, no significant adverse effects were observed in mice injected i.v. or i.p. with as many as 5×10^7 CL (data not shown). Evidence of autoimmune or GVH disease was not noted in any treated mice. Additionally, there have been no reports of significant side effects from adoptive transfer of fresh cells from tumor-immune mice, cultured cells from tumor-immune mice, or cells from tumor-immune mice grown in IL2, despite infusion of large numbers of effectors (51,52,76,89,90,236,268,287). It, therefore, appears that tumor therapy with cultured CL is possible and has few adverse side effects.

4.4 SUMMARY

DBA mice have a small "natural" host reactivity to P815 cells demonstrated by increased clearance of tumor shortly after injection of tumor into normal but not tumor-bearing mice. Similarily, 7-9 days after initial tumor injection, DBA mice cleared tumor more rapidly than normal mice suggesting a host response to P815. The increased number of cells that can become CL in tumor-bearing mice following culture with IL2 and tumor also suggests that DBA mice respond, albeit poorly, to P815 tumor.

Injection of spleen cells from tumor-bearing mice cultured with IL2 and P815 cells cleared tumor directly and also augmented the delayed host response. This is probably because the cells induced by culturing with IL2 and P815 cells consisted of both CL and helper cells.

5. THERAPY WITH IL2 GENERATED CL

5.1 INTRODUCTION

Data from many of the experiments presented in chapter 4 were pooled and presented in an actuarial manner to demonstrate any effect that CL therapy may have on survival. This pooling of experiments was required to give the number of animals necessary to establish improved survival times. Optimal routes, times of injections and combination with other cancer treatment modalities are presented.

5.2 RESULTS

5.2.1 CRITERIA FOR TREATMENT OF I.P. P815 TUMOR

The optimal conditions for improving survival times of mice receiving large doses of i.p. P815 cells were determined in a single experiment (Table 30). CL could not be injected prior to the injection of tumor. CL were effective if injected 2 hours or 48 hours after the tumor. CL must be injected into the i.p. tumor site as i.v. CL alone were not effective. Either the CL or the mice could be irradiated with only slight changes in the efficacy of the CL. Survival times correlated with the number of CL injected.

5.2.2 THERAPY OF I.P. P815 TUMOR

Data from tumor clearance experiments were pooled to provide an assessment of the ability of CL to improve survival times of mice given large doses of P815 cells. Each mouse received $1x10^6$ P815 cells i.p. on day 0 and day 7. CL given i.p. 2 hours later on day 0 improved survival times from 18±3 to 26±5 days (Figure 17). CL given 1-5 days after the tumor increased survival times from 24±5 days to 31±15 days for those mice dying from the tumor. Five percent of the treated mice survived the tumor (Figure 18).

Mice given a single i.p. injection of $1x10^3$ to $1x10^4$ P815 cells demonstrated significantly increased survival following a single i.p. injection of $1x10^7$ CL 2 hours after the tumor (Figure 19) or 1-3 days following the tumor (Figure 20). Median survival times were 23 days for untreated mice vs 31 days for treated mice. Ten percent of treated mice survived long term. This demonstrates the efficacy of CL therapy in treatment of

Table 30

Criteria for Successful therapy of i.p. P815

CL Injection				Survival (days)
Number	Location	Day	Irradiated CL	
A. Normal Mice				
0	-	-	-	18±1
3×10^7	i.p.	-2	-	18±5
3×10^7	i.p.	0	-	26±1
3×10^7	i.p.	+2	-	28±4
3×10^7	i.p.	0	+	25±3
1×10^7	i.p.	0	-	24±2
3×10^6	i.p.	0	-	22±7
3×10^7	i.v.	0	-	18±1
B. Irradiated mice				
0	-	-	-	13±2
3×10^7	i.p.	0	-	36±3

CL were generated from P815-bearing DBA mice by culture for 5 days. Mice had received 1×10^4 P815 cells 21 days previously. The killing activity was 160×10^{-8}. Cells were irradiated 2,000 rads on day of use. Mice were irradiated 600 rads two days prior to use. CL injections unless otherwise indicated were 2 hours following i.p. injection of 1×10^6 ^{131}I-IUdR-labeled P815. All mice received a second injection of 1×10^6 ^{131}I-IUdR-labeled P815 on day 7.

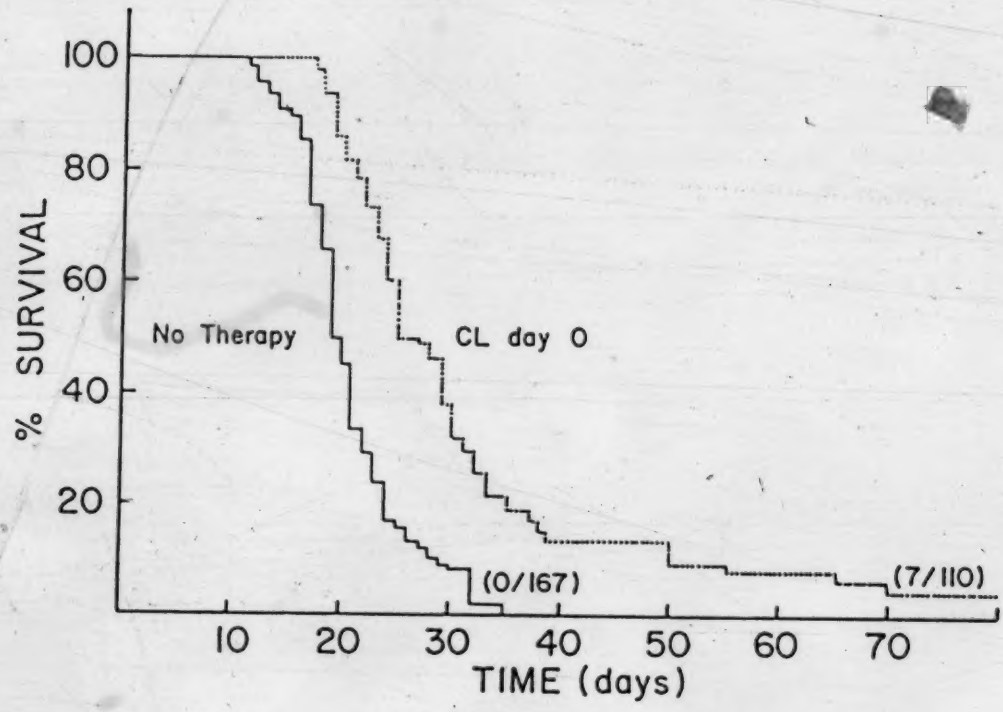

Figure 17: Therapy of high dose P815 cells i.p.: Day 0.

DBA mice, 167, received 1×10^6 P815 on day 0 and day 7 (——). DBA mice, 110, received between 1×10^6 and 3×10^7 CL i.p. 2 to 4 hours later (------). CL were generated from DBA mice which had received 1×10^4 P815 between 14 and 21 days earlier. Spleen cells from the tumor-bearing mice had been cultured with IL2 and P815 cells for 5 days. Number of survivors is indicated in brackets.

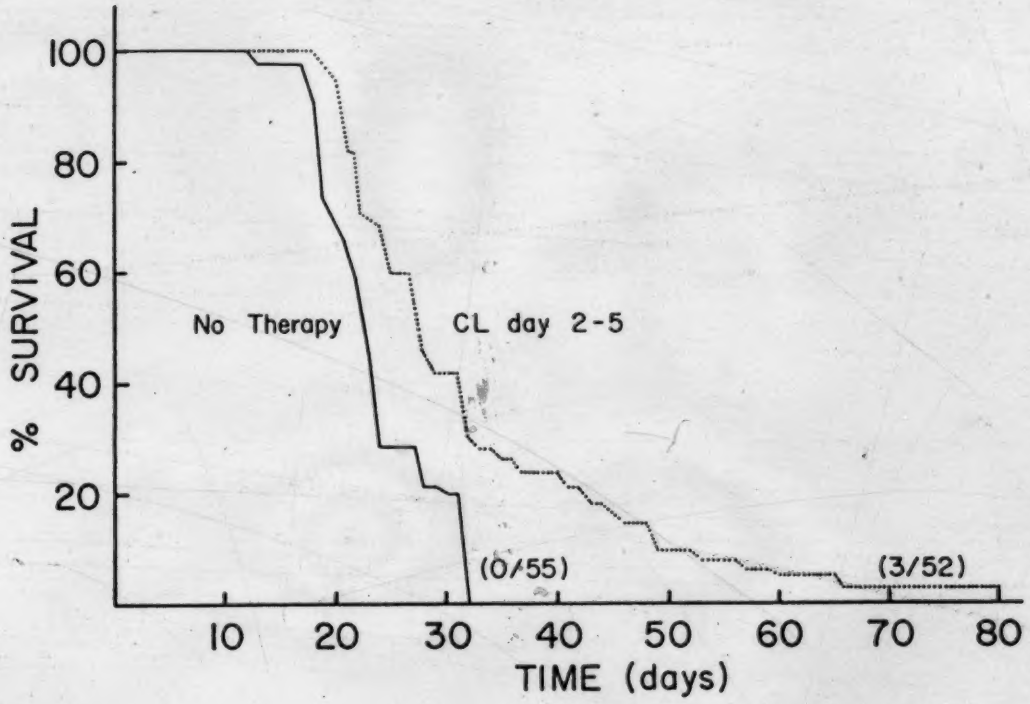

Figure 18: Therapy of high dose P815 i.p.: Delayed.

DBA mice, 107, received 1×10^6 P815 i.p. on day 0 and 7 (——). Of these mice, 52 received 1×10^7 CL on one of days 2-5 (------). CL were generated from mice which had received 1×10^4 P815 cells 14 to 21 days earlier. Spleen cells from these tumor-bearing mice were cultured for 5 days with IL2 and P815 cells. Number of survivors is indicated in the figure in brackets. Survival times for the untreated mice were 24±5 and for those treated mice dying of the tumor 31±15.

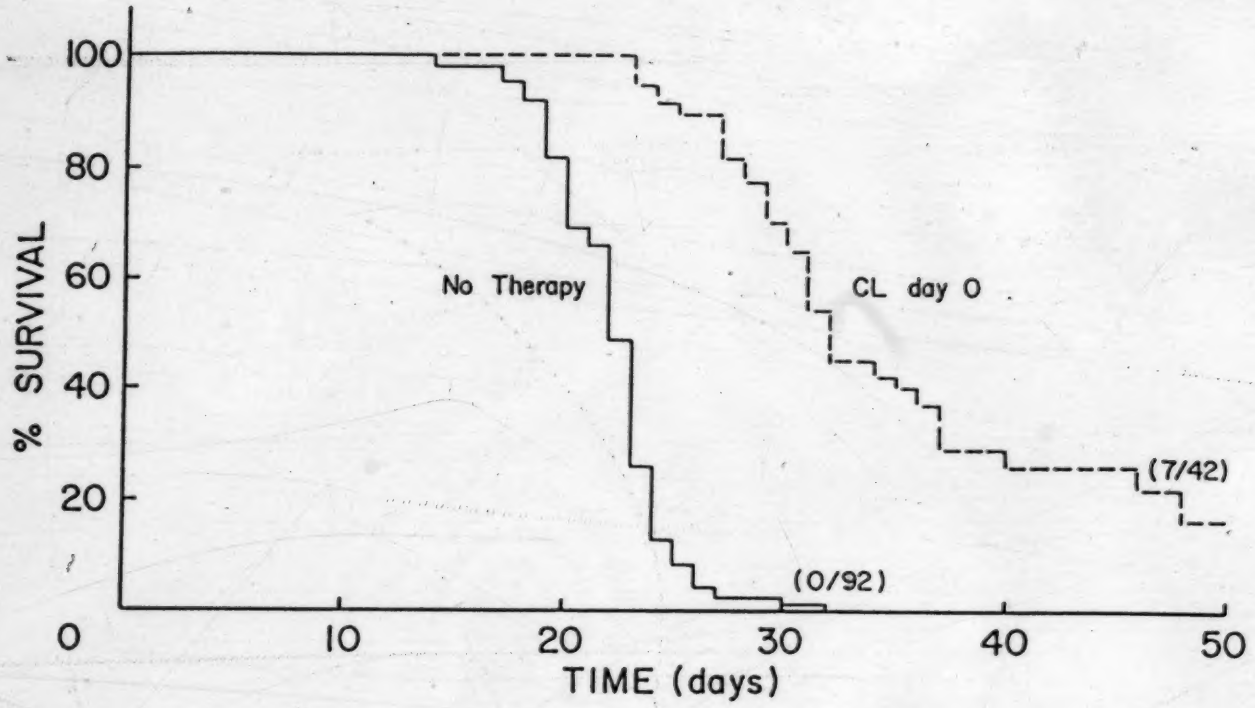

Figure 19: Therapy of low dose P815 i.p.: Day 0.

DBA mice,92, received $1x10^3$ to $1x10^4$ P815 cells i.p. on day 0 (——). Two hours later 42 of these mice received $1x10^7$ CL (------). CL were generated from tumor-bearing mice by culture with IL2 and P815 cells. Number of survivors is indicated in brackets.

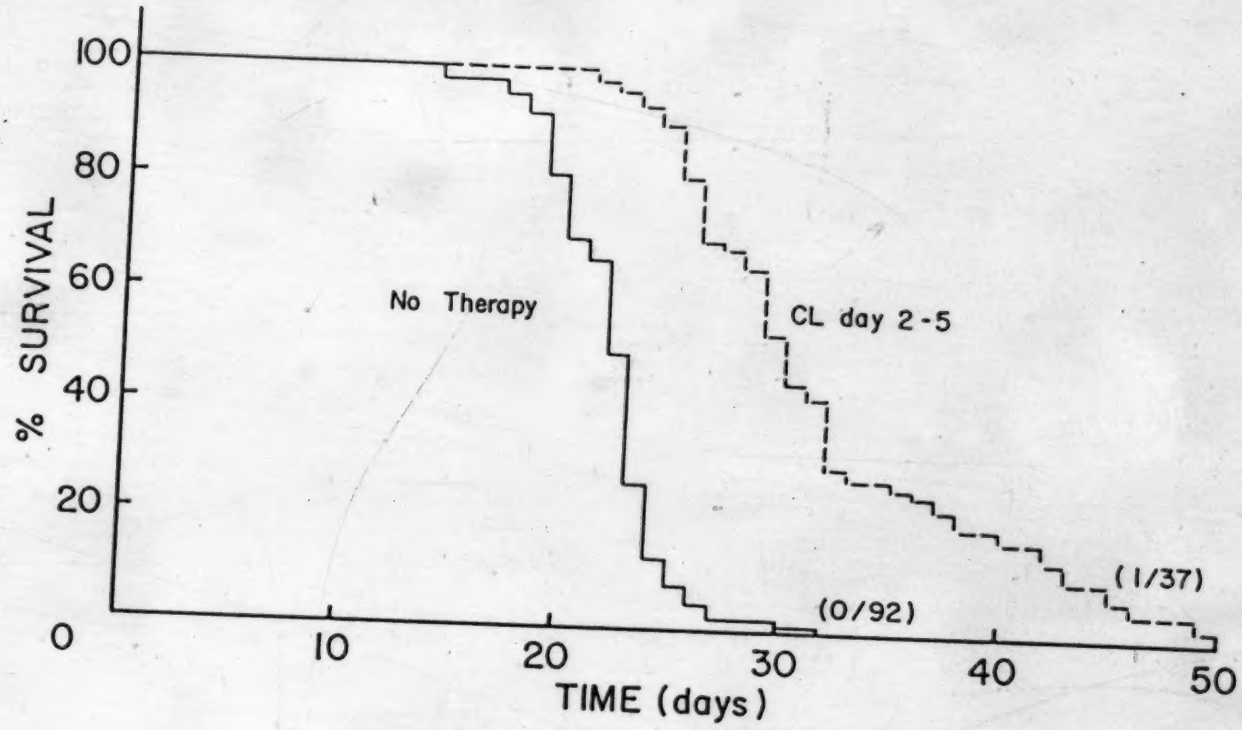

Figure 20: Therapy of low dose P815 cells i.p.: Delayed.

DBA mice, 129, received $1x10^3$ to $1x10^4$ P815 cells on day 0 (——). Of these mice, 37 received $1x10^7$ CL 2 to 5 days later (------). CL were generated from tumor-bearing mice by culture with IL2 and P815 cells. The number of survivors is presented in brackets.

early tumors. CL given on days 6-8 did not improve survival times (data not shown).

5.2.3 REPEATED CL THERAPY IS MORE EFFECTIVE

CL therapy of i.p. P815 tumor increased survival times. Still, few mice survived the tumor. To determine if repeated CL injections would enhance survival, mice, with $1x10^3$ P815 cells injected i.p., were treated with either one injection of $1x10^7$ CL (3 days after tumor injection) or with four injections of $1x10^7$ CL (3, 4, 6, and 7 days after tumor injection). CL improved survival times from 24±2 days for untreated mice to 28±2 days for a single treatment, and to 36±6 days for multiple treatments (Figure 21). Repeated therapy still did not increase cure rates. Multiple injections of CL decreased tumor burden and increased survival times but did not result in the death of all tumor cells injected into the mice.

5.2.4 TREATMENT OF SOLID TUMORS

About 40% of the mice, treated with CL i.v. and s.c., survived s.c. tumor (Figure 22). Those mice which died of the tumor did not demonstrate significantly increased survival times. Either CL were capable of eradicating s.c. tumor, totally, or did not significantly improve survival times.

5.2.5 COMBINED SURGICAL TREATMENT AND I.V. CL TREATMENT CAN RESULT IN 100% SURVIVAL

P815 and L1210 grow very rapidly and metastasize early. This makes attempts at combining surgery and CL therapy difficult. Therefore, a slower growing tumor, CaD2, was chosen as a model. This tumor grows more slowly than either P815 or L1210 with $1x10^4$ CaD2 cells s.c. causing death in about 32 days. Significant CL activity can be developed in large scale cultures of spleen cells from CaD2 tumor-bearing mice only in about 50% of experiments compared to 80-90% for P815 tumor-bearing or L1210 tumor-bearing mice (data not shown). When CL activity was obtained, it was effective *in vivo* (Figure 23). Surgery or CL therapy i.v. and into the tumor site was capable of increasing the survival time of mice with large tumor masses (10 g). Without treatment, mice died 8±1 days later while surgery increased survival to 23±6 days. CL therapy i.v.

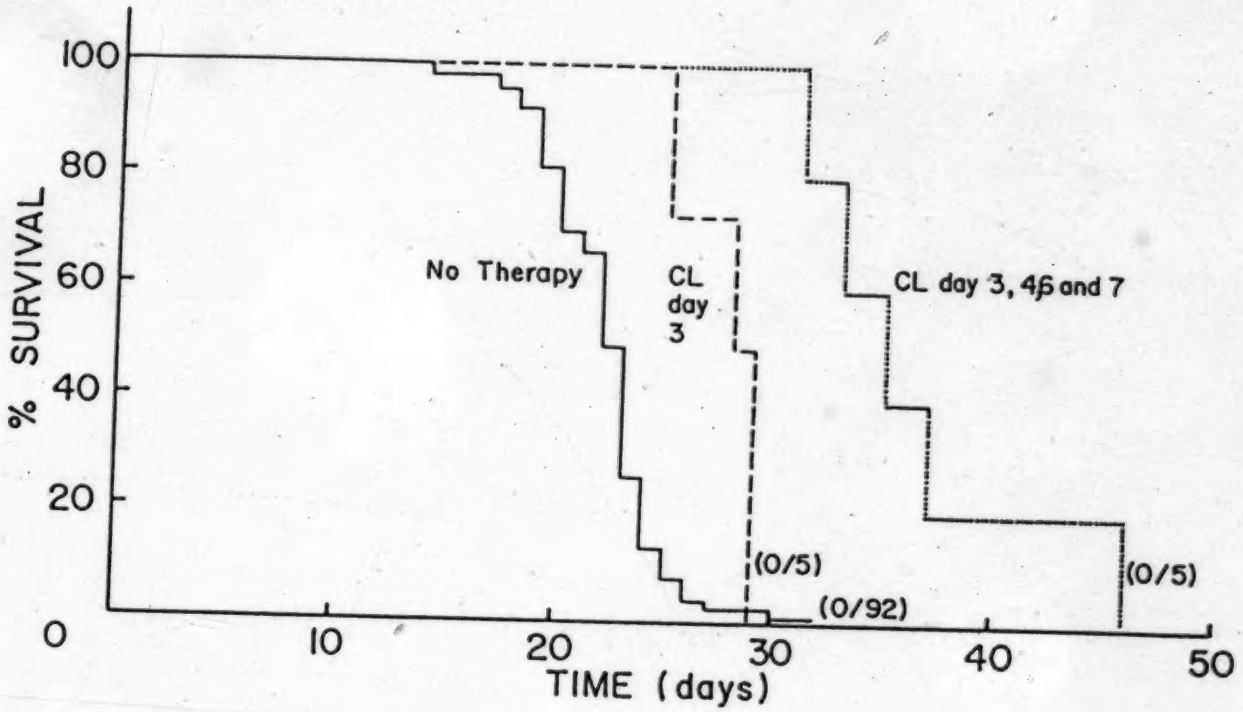

Figure 21: Therapy of low dose tumor i.p.: Repeated CL injections.

DBA mice, 102, (pooled experiments) received 1×10^3 to 1×10^4 P815 cells i.p. (——). One group of 5 mice received 1×10^7 CL on day 3 only (------). Another group of 5 mice received CL on days 3,4,6,and 7 (.....). Number of survivors is indicated in brackets. Survival times were 24±2 days for untreated mice (this experiment not pooled controls), 28±2 for one injection of CL and 36± 6 for multiple injections of CL.

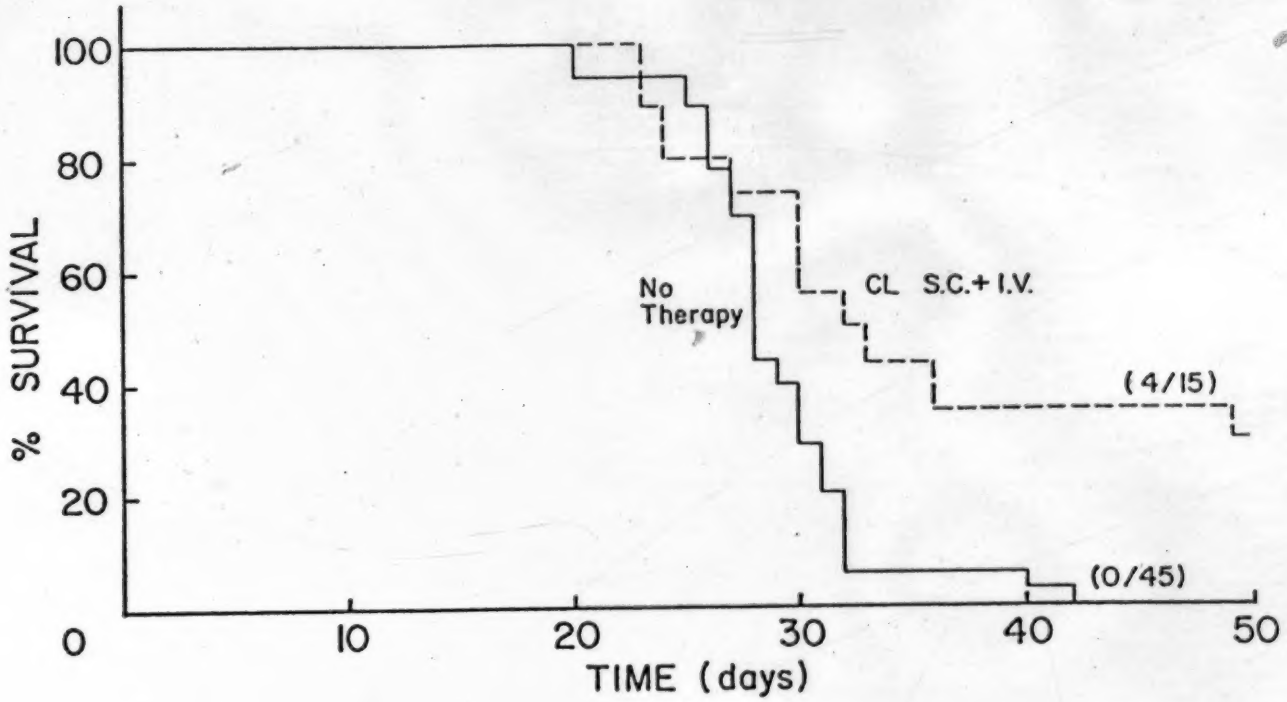

Figure 22: Therapy of s.c. P815 tumor.

P815 cells, 1×10^3, were injected s.c. into 60 DBA mice (——) (pooled experiments). Of these mice, 15 received 1×10^7 CL both i.v. and s.c. 2 hours later (------). CL were generated from DBA mice which had been injected with 1×10^4 P815 19 days earlier. CL i.v. alone did not effect survival times or result in any long term survivors (15 mice, data not presented). CL s.c. alone did not effect survival time of mice dying of the tumor but one mouse did survive long term (15 mice, data not shown). Numbers of surviving mice are indicated in brackets in the figure.

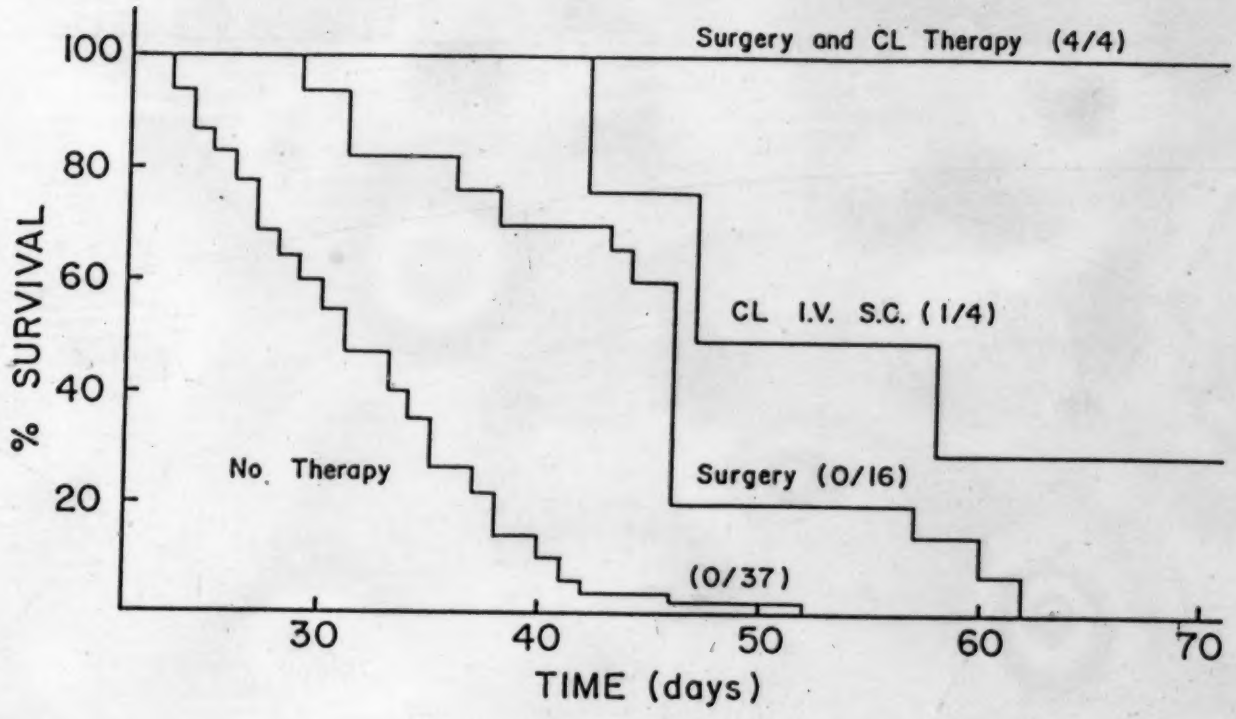

Figure 23: Combined surgery and CL therapy of mice with CaD2 tumor.
CaD2, $1x10^4$, were injected s.c. into 37 DBA mice (no therapy). One group of 16 mice was treated on day 23 after tumor injection by surgery alone (surgery). One group of 4 mice was injected with $1x10^7$ CL i.v. and s.c. on day 23 (CL i.v. s.c.). One group of 4 mice had tumor surgically removed and received $1x10^7$ CL i.v. on day 23 (Surgery and CL therapy).
CL were generated from mice which had received $1x10^4$ CaD2 cells 18 days previously. Spleen cells were cultured with CaD2 cells and IL2 for 5 days.
None of the untreated or surgically treated mice survived the tumor. One of the mice treated with CL i.v. and s.c. survived. All of the mice treated with surgery and CL i.v. survived and remained tumor immune.
On day 23, when therapy was performed, tumors were approximately 10 gm in weight. Average mouse weight was 23 gm. All surgically treated mice died of metastases rather than of local recurrence.

and into the tumor (no surgery) increased survival to 25±8 days with one mouse remaining tumor free for greater than one year. This was surprising considering the size of the tumor mass. Surgically treated mice died of metastases rather than of local recurrence.

Surgery and i.v. CL therapy were synergistic. All mice receiving this treatment remaining tumor-free and tumor-resistant for the life span of the mice. Although this experiment must be considered as preliminary, the results of this therapy of an established tumor composing one-third of the total mass of the mouse, with cells derived from tumor-bearing mice, exhibits great promise.

5.3 DISCUSSION

Adoptive transfer of immunity can be assessed by Winn assays, therapy of early tumors and therapy of established tumors. Each assay measures different aspects of cell mediated immunity (19,20,37,267).

5.3.1 WINN OR TUMOR NEUTRALIZATION ASSAY

Mixing immune lymphocytes with tumor cells prior to injection removes the need for therapeutic cells to home to the tumor. This "Winn" assay seems to be an *in vivo* correlate of the *in vitro* cytotoxicity assay. This "tumor neutralization" assay may require collaboration between cell types including subpopulations of T cells (119) and T cells and macrophages (150,317). These collaborating cells could be recruited from the host by immune lymphocytes (250,317). The relevance of Winn assays to possible therapy of human tumors is very questionable.

Lymphocytes from normal mice (15,37,161,265,289) and from tumor-immune mice (22,44,45,119,138,250,317) can mediate tumor protection in a Winn assay. Lymphocytes cultured from normal or tumor-bearing mice mice were not effective if injected into different sites or at different times from the tumor cells (37,105,260,267), suggesting that the tumor neutralization assay may not be a good model for immunotherapy techniques (267).

CL lines growing in IL2 are effective in tumor neutralization assays but not in improving survival times of mice with established tumors (110,163,291). They are also not able to home to tumor cells (110,163,291). The failure of cultured CL to clear

established tumors could be due to failure of the CL to survive *in vivo* (52, 163,268), to failure to home to the tumor (60, 190), or to lack of helper cells (129). These CL may be active if injected with exogenous IL2 (52,89,90,268).

5.3.2 TREATMENT OF EARLY TUMORS

Following culture with IL2 and tumor, lymphocytes from tumor-bearing mice cleared tumor (Figure 6,7) and augmented the response of normal DBA mice to the P815 tumor (Figure 10,11,12) (19,223,344). Injection of CL and tumor into the peritoneal cavity, even at different times, may be similar to the Winn assay in that the CL do not have to home to the tumor. It may be relevant to the treatment of human ovarian carcinoma in that almost all of the ovarian tumor is located on surfaces within the peritoneal cavity and in ascitic fluid. CL, injected into the peritoneal cavity, would not have to home to the ovarian tumor.

The injection of effector CL along with a population of cells capable of inducing host responses consistantly improved the survival times of mice given high or low doses of P815 cells i.p. (Figure 16, 18, 19, 20, 21). CL were effective from day 0-5, had virtually no effect from day 6-8 and, without additional therapy, had little effect thereafter (data not shown) (19,236,344). Repeated injections of CL decreased residual tumor and improved survival times (Figure 22, 344).

CL injected i.v. were synergistic with CL injected into s.c. tumors in the treatment of early tumors (Table 19, Figure 23). This synergism was probably through the effect of the i.v. CL in clearing i.v. metastases rather than by direct homing to the small s.c. tumor.

5.3.2.1 EFFECTOR CELLS FROM NORMAL MICE

Spleen cells from normal DBA mice cultured with IL2 and P815 cells were effective in improving survival times of mice with i.p. P815 if treatment started on day 0 (344). Repeated injections of cultured cells were more effective than a single injection. The same cells were not effective in improving survival times of mice with established tumors (i.e. if given 8 to 15 days after initial tumor injection) (344).

5.3.2.2 EFFECTOR CELLS FROM IMMUNE ANIMALS

Allogeneic immune cells (62) and even xenogeneic cells (28,358) are capable of transferring immunity to mice early in tumor growth. The efficacy of the therapy, particularily with allogeneic immune cells, is limited by severe GVH disease. In this study, GVH disease may have been the effector mechanism in improving survival rather than a specific anti-tumor effect (86).

Intravenous injection of immune syngeneic spleen cells can delay growth of intradermal tumors (28,287) and i.p. tumors (21) if given on the same day as tumor cells. Immune syngeneic cells were more effective than immune allogeneic or xenogeneic cells (28). *In vitro* culture of the immune cells increased their efficacy (287).

Intravenous injection of immune spleen cells prevented growth of i.p. tumors on day 1-3 but not thereafter in normal mice (19). At later times immune cells were only effective in immunosuppressed mice (19,20).

5.3.2.3 EFFECTOR CELLS FROM TUMOR-BEARING ANIMALS

Our treatment protocol of mice with early tumors is unique in that the effector cells were developed by culture from tumor-bearing mice. Other reports of treatment with similar cells are not available.

5.3.3 TREATMENT OF ESTABLISHED TUMORS

CL were effective in clearing i.p. tumor from mice which also had large s.c. tumors (Figure 15). Because P815 was such a virulent and rapidly growing tumor, CaD2 was examined as an alternative model for immunotherapyof established tumors.

The choice of CaD2, a spontaneous mammary adenocarcinoma, as a tumor model may have been particularily propitious since CaD2 is non-immunogenic even in allogeneic mice (176,283). It grows as well in allogeneic mice as in syngeneic mice (data not shown). CaD2 does not generate concomitant immunity in either syngeneic or allogeneic mice (75). The development of concomitant immunity seems to be a precursor to the development of suppressor cells in mice with progressive tumors (75). CaD2 may not induce suppressor cells. CL therapy, therefore, may not have to overcome suppressor cells.

CL injected into large CaD2 tumor masses were as effective as surgery in improving survival times of mice (Figure 23) (CL injection into s.c. P815 masses did not

improve survival times but neither did surgery, data not shown). One mouse, despite having an approximately 10 gram tumor mass, survived following a s.c. injection of CL alone. All four mice treated by surgery combined with i.v. CL survived the tumor (Figure 23).

Although therapy of established CaD2 tumors must be considered preliminary due to the small numbers of treated animals, the improved survival times and cures were clearly different from the large numbers of control animals. Of 37 untreated CaD2 tumor-bearing mice and 16 surgically treated mice, none survived the tumor. One out of four mice treated with CL i.v. and s.c. and four out of four treated with surgery and CL i.v. survived.

5.3.3.1 TREATMENT OF IMMUNOSUPPRESSED ANIMALS

Other techniques have been used to abrogate the effect of suppressor cells *in vivo*. Generally animals are rendered immunodeficient by treatment with cyclophosphamide (51,52,223,268) or treatment with radiation (20,74,76,87,89,90,268). Treatment of these immunodeficient animals with cells from immune animals either directly from the immune animals (51,52,223) or with cells cultured from immune animals (76,89,90,129,268) established many of the criteria for immunotherapy of established tumors. Since irradiation decreases its effectiveness, the effector cell must persist and divide *in vivo* (51,76,127). The tumor grows for a period of time in the presence of the injected effector cell and then regresses due to recruitment of host cells (20,87,126).

Although cultured cells seem more effective in improving survival times than cells directly out of immune mice, cultured cells do not improve the cure rate (44,45,48,49,51). Addition of IL2 to the treatment regimen improves the cure rate (51,52,268). Cultured cells are more effective when injected directly into the tumor than when injected i.v. Cultured cells injected i.v. are less effective than non-cultured immune cells (48,49) suggesting that cultured cells do not home appropriately or survive long enough *in vivo* to be effective. Culture with antigen alone (46,87) or with IL2 (51,76,89,90,268) can improve the efficacy of immune cells.

Although the effector cell inducing host responses is probably a helper cell rather than a CL (89,127,129,223), CL lines expanded in IL2 can be effective in improving

survival if IL2 is injected i.p. along with the CL (52,89,90,268).

The effector cell is tumor-specific (75,88,128) and H2 restricted (128).

5.3.4 TREATMENT OF ESTABLISHED TUMORS IN NORMAL ANIMALS

5.3.4.1 TREATMENT WITH CELLS FROM TUMOR-IMMUNE ANIMALS

Peritoneal exudate cells from tumor-immune guinea pigs were effective in i.v. treatment of ascites line-1 tumor cells in strain 2 guinea pigs (353). Immune peritoneal exudate cells, $1x10^8$, were effective in stopping tumor growth up to 12 days after intramuscular tumor injection (353). Repeated injections of immune cells were more effective than single injections. Immunity was tumor-specific. Lymphocytes mediating DTH seemed to be responsible for prolonging survival (354). Surgical removal of the original tumor implant allowed therapy of line-1 metastases in mice with 14-day old tumors (290). Immunity was strain and tumor-specific (290). Intralesional BCG therapy increased the efficacy of the immune peritoneal exudate cells (290).

Spleen cells from tumor-immune rats cultured *in vitro* with the immunizing tumor were effective in treating normal animals 6 days after i.v. tumor injection (87). The same immune cells were more effective in animals given 450 rad of radiation (87). The effector cell in irradiated animals was a noncytolytic cell, probably a helper cell. With irradiated animals, therapy could be delayed to as late as 18 days after tumor injection. Multiple doses totalling $7.5x10^8$ cells were necessary to cure these animals (88). This suggests that irradiation abrogates suppressor effects thus increasing the efficacy of the therapy.

5.3.4.2 TREATMENT WITH CELLS FROM NORMAL MICE

Spleen cells, from normal mice, cultured on syngeneic 3LL tumor monolayers were effective in clearing metastatic tumor in syngeneic mice (322). The initial foot pad tumor was removed 7 days after tumor injection. Therapy immediately followed. Cultured lymphocytes, $1.4x10^7$, improved survival from 40% to 70%. Lymphocytes from tumor-bearing mice similarily sensitized were ineffective (322).

5.3.4.3 TREATMENT WITH CELLS FROM TUMOR-BEARING MICE

Our results (Figure 23), although preliminary, constitute the only reported successful therapy of established tumors with lymphocytes generated from mice with established tumors. It is this system which best parallels any possible therapy of human tumors.

6. CONCLUDING DISCUSSION

6.1 GENERATION OF CL FROM TUMOR-BEARING MICE AND HUMANS

Syngeneic tumor cells, either methylcholanthrene-induced (P815, L1210, RI) or spontaneous (CaD2, A.F.), seem to contain antigens recognizable by the immune system. It seems likely that the methylcholanthrene-induced tumors elicit a population of suppressor cells which prevent IL2 release. The antigenic configuration of the spontaneous tumors may be such that IL2 is not released. Whatever the mechanism, the addition of exogenous partially purified IL2 to the cultures results in the generation of CL responses against these tumors from normal or tumor-bearing humans and normal or tumor-bearing mice.

There seems to be an increased population of precursor cytolytic T cells in the spleens of tumor-bearing mice as compared to normal mice. This population may have already recognized the tumor antigens and, following limited proliferation, is blocked at a stage of development requiring IL2 or a factor contained in the IL2 preparations for further growth. Culture with tumor is also necessary for the full expression of anti-tumor activity.

Tumor-bearing mice contain a population of specific CL inducible by culture with IL2 and tumor and a population of less specific cytolytic cells inducible by culture with exogenous IL2 alone. The maximum cytolytic activity of the non-specific population appeared at three days which is two days earlier than the maximal activity of the tumor-specific CL. This is still much later than the time course expected for interferon-induced NK activity. Both populations of cells carry phenotypic markers typical of cytotoxic lymphocytes but the differentiation between cultured cytotoxic lymphocytes and cultured NK cells is very difficult.

The lack of IL2 activity in tumor-bearing mice could result from a number of mechanisms. The relative importance of each has yet to be determined. Possible mechanisms include: suppressor cells preventing IL2 release or action, suppressor cells preventing acquisition of IL2 receptors, and failure of IL2 release due to antigenic configurations non-stimulatory for helper T cells.

Although the term IL2 has been used throughout this thesis to describe the factor active in CL generation, IL2 may not be the active factor or may act in concert with many other factors. The IL2 used in most of these experiments was only partially purified by gel exclusion chromatography and ion exchange chromatography. Interferon was known to be present in most of the preparations. IL2, purified by chromatography over a S-300 column equilibrated in SDS, was effective in the generation of CL against syngeneic tumors but even this preparation was calculated to be less than one percent pure (data not shown). The ability of the responding cells to produce many other factors, including interferon and the terminal differentiation factors, makes it impossible to attribute the activity or activities inducing CL to any one factor. It can only, therefore, be stated that partially purified IL2 is capable of inducing tumor-specific CL from tumor-bearing animals.

Throughout this thesis, the populations of cells generated *in vitro* by culture with IL2 have been referred to as CL. This population contains not only CL but probably NK cells as well as helper cells directed against the tumor. The choice of the DBA tumor systems had the drawback of not allowing separation of helper T cells from CL on the basis of Ly markers so indirect means of determining which was the effective cell type had to be used. The *in vivo* activity of the IL2-induced populations seemed to result from the activity of both the cytolytic cells, including the CL and NK cells, and a population of helper T cells. The tumor-bearing host played an important role in tumor clearance, probably through activity induced by the cultured helper T cells.

Since helper T cells may be more important than CL in therapy of tumor-bearing mice, an *in vitro* assay of helper activity may be a more appropriate screening assay for following the effect of IL2 than the CL assay used. Unfortunately, such an assay is not readily available. The *in vitro* cytotoxicity assay, though, provided an easy, reproducible, quick assay for the screening of many *in vitro* culture conditions and, until *in vitro* helper cell assays are available, provides a good screen for IL2-generated CL and IL2-generated helper activity.

One of the problems encountered in this research, that of a source of IL2 active on human cells, has been solved by other workers. Large amounts of IL2, active on human cells, can be obtained from cultures of Jurkat (106) or MLA 144 cells (253). The isolation of the cDNA for IL2 suggests that IL2 generated from the human gene inserted into

bacteria or yeast will soon be availabel (313). The production of a monoclonal antibody, which after binding to a column matrix, binds IL2, will aid in the rapid purification of IL2 (K. Smith, personal communication).

6.2 TUMOR THERAPY SYSTEMS

To parallel as closely as possible human malignant disease, CL were generated from syngeneic tumor-bearing mice and humans. Murine CL were developed against syngeneic methycholanthrene-induced P815, a mastocytoma; L1210, a lymphocytic leukema; RI, a T lymphoma; and the spontaneous mammary adenocarcinoma, CaD2. Human CL active against autologous ovarian cystadenocarcinomas were developed using similar techniques.

The CL-containing population killed tumor directly *in vivo* and resulted in a delayed host response, probably due to helper cells in the CL population. The CL-containing population was able to home, although poorly, to large s.c. tumors or to i.v. tumor implants. The CL consistantly improved survival times of i.p. tumor-bearing mice and combined CL i.v. and s.c. were able to cure a significant proportion of mice with small s.c. tumors. Despite any suppressive effects present in animals with established tumors, CL could clear tumor from tumor-bearing animals. In preliminary studies, CL combined with surgery was able to cure 100% of mice with large s.c. CaD2 tumors.

6.2.1 POSSIBLE HUMAN IMMUNOTHERAPY

Several aspects of the previously reported tumor immunotherapy systems limit their possible application to human patients. In the papers by North (19,20,207,222,223), F1 mice were used because syngeneic mice did not develop the appropriate immune cells (207). The tumors used by Rosenberg (76,268) would grow only in irradiated mice (76) or would grow only if injected i.p. and not s.c. in normal mice (268) suggesting that these tumors may have abnormal growth patterns and may therefore not be appropriate for human models. Similarily the tumor used by Fefer (FBL-3) (46,47,86,129) would grow at low dose i.p. but only at very high doses s.c. (45).

Even in the immunosuppressed animals, treatment was usually early (Fefer - day 4, North - day 4-6, Fernandez-Cruz - day 6). This is before suppressor cells can be detected

in vitro (278,308) although they can be detected *in vivo* (20,207). The cells used by Fernandez-Cruz (87) were effective up to 18 days after tumor injection if given in multiple doses totalling 7.5×10^8 cells. Cells which were effective in mice rendered immunodeficient by irradiation or cyclophosphamide treatement were less effective (87) or ineffective (19,129) in normal mice. Our technique of using cells developed from tumor-bearing mice in the therapy of tumor-bearing mice abrogates some of these problems.

6.3 FUTURE PROSPECTS

Techniques are already available for culturing large numbers of CL directed against autologous tumors. Although these CL probably will not induce host responses and may home poorly to tumor, they may be effective in killing tumor if injected into tumor masses. Ovarian cancer is particularily amenable to this form of therapy as most, if not all, malignant cells are in the peritoneal cavity and could possibly be reached by i.p. CL injection. The short half-life and efficacy of CL populations *in vivo* may be augmented by i.p. administration of IL2; once again a possible therapy for human ovarian cancer.

The use of primed CL to kill i.p. tumor may bypass the suppressor activity involved in progressive tumor growth (20). Most of the reported suppressor cells from tumor-bearing animals are effective at the initiation of cytolytic responses *in vitro* (278,308,309) or *in vivo* (19,20). Tumor-induced suppressor cells are also specific (75) with a titratable effect on immune spleen cells (74). Suppressor cells arise between day 5 (19), day 6 (75), and day 9 (207) depending on tumor type and suppressor assay. Although some tumor-bearing mice do contain suppressor cells capable of preventing action of committed CL (96), this does not seem to be the general rule (219,267). Additionally, use of committed CL may abrogate the possibility of tumor enhancement by injection of suppressor cells in fresh or cultured cells (18,88,289,322,325,326). The worry about injection of suppressor cells in cultured cell populations may not be particularily pertinent in that animals with established tumors probably have well established suppressor systems. Although the effector cell in inducing host responses may be a helper cell rather than an effector CL, therapy with CL may have a role to play in the therapy of certain accessible tumor types such as ovarian cancer.

The cell effective in inducing host responses seems to be a tumor-specific helper cell. Improved techniques for culture and cloning of helper cells may allow therapy with large numbers of tumor-specific helper cells. Since the activity of this cell may be blocked by suppressor cells present in the cancer patient, techniques for removing these suppressor cells may be needed. Quite probably the chemotherapy and radiation therapy that cancer patients already receive significantly decreases the activity of these suppressor cells. The effect of the therapies already used in human malignancies on this suppressor population needs to be assessed in animal models. Whether exogenous IL2 or helper cells will reconstitute the suppressor cells needs to be determined.

CL therapy seems to be most effective if used in mice in which the majority of the tumor mass has been removed by surgery, chemotherapy or radiation therapy. The appropriate timing of these various modes of therapy needs to be determined in animal models and applied to possible human therapy trials.

Prior to initiation of CL immunotherapy in humans, animal models should be developed to allow study of combining this treatment with chemotherapy, radiation therapy and surgery as already used in the human. Because of the differences in behavior between "spontaneous" and viral or chemically induced tumors, the therapy of spontaneous tumors would be a preferential model. Efficacy of this therapy must be established in animals with established tumors. Concomitantly, systems should be established for the growth, cloning and storage of appropriate effector cell populations from human patients.

BIBLIOGRAPHY

1 Aarden, L.A., et al., 1979. Revised nomenclature for antigen-nonspecific T cell proliferation and helper factors. J. Immunol., 123:2928.

2 Alaba, O., and I.E. Bernstein 1978. Tumor-growth suppression *In vivo*: Cooperation between immune lymphoid cells sensitized *in vitro* and nonimmune bone marrow cells. J. Immunol., 120:1941.

3 Altman, A., T.E. Bechtold and D.H. Katz 1979. Manipulation of cytotoxic T lymphocyte responses to syngeneic tumors 1. Hapten-reactive helper T lymphocytes augment tumor-specific CL responses *in vitro*. J. Immunol., 122:2484.

4 Altman, A., and D.H. Katz 1980. The induction of autoreactive T lymphocytes by allogeneic effect factor (AEF): Relevance to normal pathways of lymphocyte differentiation. Immunol. Reviews, 51:3.

5 Altman, A., A.M. Theofilopoulous, R. Weiner, D. Katz and F. Dixon 1981. Analysis of T cell function in autoimmune murine strains: Defects in production of and responsiveness to Interleukin 2. J. Exp. Med., 154:791.

6 Altman, A., A. Sferruzza, R. G. Weiner, and D.H. Katz 1982. Constitutive and mitogen-induced production of T Cell Growth Factor by stable T cell hybridoma lines. J. Immunol., 128:1365

7 Andrews,G.A., C.C. Congdon, C.L. Edwards, N. Gengozian, B. Nelson, and H. Vodpick 1967. Preliminary trials of clinical immunotherapy. Cancer Res. 27:2535.

8 Andrus, L., S.J. Prowse and K.J. Lafferty 1980. Interleukin 2 production by T cells: Triggering of T cell activation and lymphokine release. Behring Inst. Mitt., 67:61.

9 Bach, F.H., M.L. Bach, M. Segall, K. Stouberzier, P.M. Sondel, B.J. Alter, and M.L. Bach 1973. Cell-mediated immunity: Separation of cells involved in recognitive and destructive phases. Science 180:403.

10 Bach, F.H., B.Leshem, O.J. Kuperman, H. Bunzendahl, B.J. Alter and J.M. Zarling 1982. Allosensitization to obtain anti-tumor immunity. Prog. in Cancer Res. and Therapy, 22:7.

11 Baker, P.E., S. Gillis, M.M. Ferm and K.A. Smith 1978. The effect of T cell growth factor on the generation of cytolytic T cells. J. Immunol., 121:2168.

12 Baker, P.E., S. Gillis and K.A. Smith 1979. Monoclonal cytolytic T cell lines. J. Exp. Med., 149:273.

13 Baker, P.E., J.V. Fahey and A. Munck 1981. Prostaglandin inhibition of T cell proliferation is mediated at two levels. Cell Immunol., 61:52.

14 Baldwin, R.W., 1977. Immune surveillance revisited. Nature 270:557.

15 Bartlett, P.F., B.A. Fenderson and M. Edidin 1978. Inhibition of tumor growth mediated by lymphocytes sensitized in vitro to a syngeneic murine teratocarcinoma 402AX. J. Immunol., 120:1211.

16 Ben-Nun, A., and I.R. Cohen 1982. Spontaneous remission and acquired resistance to autoimmune encephalomyelitis (EAE) are associated with suppression of T

cell reactivity: Suppressed EAE effector T cells recovered as T cell Lines. J. Immunol. 128:1450.

17 Berczi, I., and A.H. Sehon 1977. Tumor inhibition by effector cell cultured from progressing sarcomas. Immunol. Commun., 6:617.

18 Berczi, I., and A.H. Sehon 1979. Effector and suppressor lymphoid Cells in tumor-bearing guinea-pigs. Int. J. Cancer, 23:274.

19 Berendt, M.J., R.J. North and D.P. Kirstein 1978. The immunological basis of endotoxin-induced tumor regression. Requirement for T-cell-mediated immunity. J. Exp. Med., 148:1550.

20 Berendt, M.J., and R.J. North 1980. T-cell-mediated suppression of anti-tumor immunity. An explanation for progressive growth of an immunogenic tumor. J. Exp. Med., 151:69.

21 Bernstein, I.D. 1977. Passive transfer of systemic tumor immunity with cells generated *in vitro* by a secondary immune virus-induced lymphoma. J. Immunol., 118:122.

22 Bernstein, I.D., E.F. Cohen, and P.W. Wright 1977. Relationship of cellular proliferation and the generation of cytotoxic cells in an *in vitro* secondary immune response to syngeneic rat lymphoma cells. J. Immunol., 118:1090.

23 Bleackley, R.C., B. Caplan, C. Havele, R. G. Ritzel, T.R. Mosmann, J.F. Farrar and V. Paetkau 1981. Translation of lymphocyte mRNA into biologically-active Interleukin 2 in oocytes. J. Immunol. 127:2432.

24 Bleackley, R.C., C. Havele and V. Paetkau 1982. Cellular and molecular properties of an antigen-specific cytotoxic T lymphocyte line. J. Immunol. 128:758.

25 Bonnard, G.D., K. Yasaka, and D. Jacobson 1979. Ligand activated T cell growth factor induced proliferation: Absorption of T cell growth factor by activated T cells. J. Immunol., 123:2704.

26 Bonnard, G.D., K. Yasaka and R.D. Maca 1980. Continued growth of functional human T lymphocytes: Production of human T-cell growth factor. Cell. Immunol., 51:390.

27 Borberg, H. and H.F. Oettgen 1971. Inhibition of growth of chemically induced sarcomas in syngeneic mice by immune lymphocytes. Int. J. Cancer, 9:427.

28 Borberg, H., H.F. Oettgen, K. Choudry and E.J. Beattie, Jr. 1972. Inhibition of established transplants of chemically induced sarcomas in syngeneic mice by lymphocytes from immunized donors. Int. J. Cancer, 10:539.

29 Bortin, M.M., A.A. Rimm, and E.C. Saltzstein 1973. Graft Versus Leukemia: Quantification of adoptive immunotherapy in murine leukemia. Science, 179:811.

30 Bortin, M.M., A.A. Rimm, G.E. Rodey, R.H. Giller and E.C. Saltzstein 1974a. prolonged survival in long-passage AKR leukemia using chemotherapy, radiotherapy and adoptive immunotherapy. Cancer Research 34:1851.

31 Bortin, M.M., A.A. Rimm, W.C. Rose, and E.C. Saltzstein 1974b. Graft-Versus-Leukemia. V. Absence of antileukemic effect using allogeneic H-2-identical immunocompetent cells. Transplantation, 18:280.

32 Broder, S., L. Muul and T.A. Waldmann 1978. Suppressor cells in neoplastic disease. J. Natl. Cancer Inst. 61:5.

33 Burakoff, S.J., O. Weinberger, S.H. Herrmannand, M.F. Mescher 1980a. The use of purified H-2 antigens to define the helper T cell pathway involved in cytolytic T lymphocyte induction. Behring Inst. Mitt., 67:118.

34 Burakoff, S.J., V.H. Engelhard, J. Kaufman and J.L. Strominger 1980b. Induction of secondary cytotoxic T lymphocytes by liposomes containing HLA-DR antigens. Nature, 283:495.

35 Burnet, F.M., 1967. Immunological aspects of malignant disease. Lancet 1:1171.

36 Burnet, F.M., 1970. Immunological Surveilance. Pergammon Press Oxford.

37 Burton, R.C., and N.L. Warner 1977. *In vitro* induction of tumor specific immunity. IV. Specific adoptive immunotherapy with cytotoxic T. cells induced *in vitro* to plasmacytoma antigens. Cancer Immunol. Immunother., 2:91.

38 Cantor, H., and R. Asofsky 1972. Synergy among lymphoid cells mediating the graft versus host response. III. Evidence for interaction between two types of thymus-derived cells. J. Exp. Med. 67:764.

39 Cantor, H, and E.A. Boyse 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II Cooperation between sublasses of Ly (+) cells in the generation of killer cells. J. Exp. Med. 141:1390.

40 Caplan, B., C. Gibbs and V. Paetkau 1981. Properties of sodium dodecyl sulfate-denatured Interleukin 2. J. Immunol. 126:1351.

41 Carlson G.A., and G. Terres, 1978. The effects of passive immunization with tumor-specific antiserum on the active immune response of mice to sublines of leukemia L1210. J. of Immunol. 121:1752.

42 Cerottini, J., and K.T. Brunner 1981. Cell-mediated cytotoxicity allograft rejection and tumor immunity. J. Natl. Cancer Inst. 67:131.

43 Chang, M.-P., T. Makinodan, W.J. Peterson and B.L. Strehler 1982. Role of T cells and adherant cells in age-related decline in murine Interleukin 2 production. J. Immunol. 129:1426.

44 Cheever, M.A., R.A. Kempf and A. Fefer 1977. Tumor neutralization, immunotherapy, and chemoimmunotherapy of a friend leukemia with cells secondarily sensitized in vitro. J. Immunol., 119:714.

45 Cheever, M.A., P.D. Greenberg and A. Fefer 1978. Tumor neutralization, immunotherapy, and chemoimmunotherapy of a friend leukemia with cells secondarily sensitized in vitro. II. Comparison of cells cultured with and without tumor to noncultured immune cells. J. Immunol., 121:2220.

46 Cheever, M.A., P.D. Greenberg and A. Fefer 1980a. Therapy of leukemia by nonimmune syngeneic spleen cells. J. Immunol. 124:2137.

47 Cheever, M.A., P.D. Greenberg and A. Fefer 1980b. Specificity of adoptive chemoimmunotherapy of established syngeneic tumors. J. Immunol. 125:711.

48 Cheever, M.A., P.D. Greenberg, and A. Fefer 1981a. Chemoimmunotherapy of a Friend leukemia with cells secondarily sensitized *in vitro*: Effect of culture duration on therapeutic efficacy. J. Natl. Cancer Inst., 67:169.

49 Cheever, M.A., P.D. Greenberg, and A. Fefer 1981b. Adoptive therapy of estiblished syngeneic leukemia cells primarily sensitized *in vitro*. Cancer Res., 41:2658.

50 Cheever, M.A., P.D. Greenberg, and A. Fefer 1981c. Specific adoptive therapy of established leukemia with syngeneic lymphocytes sequentially immunized *in vivo* and *in vitro* and nonspecifically expanded by culture with Interleukin 2. J. Immunol., 126:1318.

51 Cheever, M.A., P.D. Greenberg, S. Gillis, and A. Fefer 1982a. Specific adoptive therapy of murine leukemia with cells secondarily sensitized *in vitro* and expanded by culture with Interleukin 2. Prog. in Cancer Res. and Therapy, 22:127.

52 Cheever, M.A., P.D. Greenberg, A. Fefer and S. Gillis 1982b. Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by *in vivo* administration of purified Interleukin 2. J. Exp. Med., 155:968.

53 Chouaib S. and D. Fradelizi 1982. The mechanism of inhibition of human IL2 production. J. Immunol. 129:2463.

54 Clark-Lewis, I. and J.W. Schrader 1982. Biochemical characterization of regulatory factors derived from T cell hybridomas and spleen cells. II. Evidence for glycosylation of TCGF, T Cell Replacing Factor and GM-CSF. J. Immunol. 128:175.

55 Conlon, P.J., S.H. Hefeneider, C.S. Henney, and S. Gillis 1982a. The effects of Interleukin 2 on primary *in vivo* immune responses. Prog. in Cancer Res. and Therapy, 22:113.

56 Conlon, P.J., C.S. Henney and S. Gillis 1982b. Cytokine-dependent thymocyte responses: Characterization of IL1 and IL2 target subpopulations and mechanism of action. J. Immunol. 128:797.

57 Conlon, P.J., C.A. Ramthun, C.S. Henney and S. Gillis 1982c. Cytokine-dependent thymocyte responses. II. Generation of cytotoxic T lymphocytes from immature thymocytes. J. Immunol., 129:11.

58 Currie, G.A. 1972. Eighty Years of immunotherapy: a review of immunological methods used for the treatment of human cancer. British J. of Cancer 26:141.

59 Czitrom, A.A., G.H. Sunshine, T. Reme, R. Ceredig, A.L. Glasebrook, A. Kelso, and H. R. MacDonald 1983. Stimulator cell requirements for allospecific T cell subsets: specialized accessory cells are required to activate helper but not cytolytic T lymphocyte precursors. J. Immunol. 130:546.

60 Dailey, M.O., C.G. Fathman, E.C. Butcher, E. Pillemer and I. Weissman 1982. Abnormal migration of T Lymphocyte clones. J. Immunol. 128:2134.

61 Deckers, P.J., B.W. Edgerton, B.S. Thomas, and Y.H. Pilch 1971. The adoptive transfer of concomitant immunity to murine tumor isografts with spleen cells from tumor-bearing animals. Cancer Research 31:734.

62 Delorme, E.J., and P. Alexander 1964. Treatment of primary fibrosarcoma in the rat with immune lymphocytes. Lancet ii:117.

63 Delovitch, T.L., J. Watson, R. Battistella, J.F. Harris, J. Shaw and V. Paetkau 1981. In vitro analysis of allogeneic lymphocyte interaction. V. Identification and characterization of two components of allogeneic effect factor, one of which displays H-2-restricted helper activity and the other, T cell-growth factor activity. J. Exp. Med. 153:107.

64 DeLustro, F. and J.S. Haskill 1978. In situ cytotoxic T cells in a methylcholanthrene-induced tumor. J. Immunol., 121:1007.

65 Dempsey, R.A., C.A. Dinarello, J.W. Meir, L.J. Rosenwaser, M. Allegretta, T.E. Brown, and D.R. Parkinson 1982. The differential effects of human leukocyte pyrogen/lymphocyte activating factor, T cell growth factor, and interferon on human natural killer activity. J. Immunol. 129:2504.

66 Dennert, G. 1980. Cloned lines of natural killer cells. Nature 287:47.

67 Dennert, G., and J.F. Warner 1982. Cloned T cell lines with multiple functions. Prog. in Cancer Res. and Therapy, 22:221.

68 DiSabato, G., D.M. Chen and M. Altin 1978. Production of thymocyte stimulating factor by murine spleen lymphocytes: Cellular and biochemical mechanism. Cell Immunol., 35:340

69 DiSaia, P.J., F.N. Rutlege, J.P. Smith and J.G. Sinkovics 1971. Cell mediated immune reaction to two gynecologic malignant tumors. Cancer 28:1129.

70 Domzig, W., B.M. Stadler, and R.B. Heberman 1983. Interleukin 2 dependence of human natural killer (NK) activity. J. Immunol. 130:1970.

71 Dos Reis, G.A., and E.M. Shevach 1982. Effect of cyclosporin A on T cell function in vitro: The mechanism of suppression of T cell proliferation depends on the nature of the T cell stimulus as well as the differentiation state of the responding cell. J. Immunol. 129:2360.

72 Dullens, H.F.J., and W. Den Otter 1974. Therapy with allogeneic immune peritoneal cells. Cancer Res., 34:1726.

73 Duprez, V., E. Gomard and J.P. Levy 1978. Cell mediated anti-tumor response in the RLQ 1 system 1. T nature, H-2D involvement and antigenic specificity of the effector cells. Eur. J. Immunol., 8:650.

74 Dye, E.S., and R.J. North 1981. T cell-mediated immunosuppression as an obstacle to adoptive immunotherapy of the P815 mastocytoma and its metastases. J. Exp. Med., 154:1033.

75 Dye, E.S., R.J. North, and C.D. Mills 1981. Mechanisms of anti-tumor action of corynebacterium parvum. I. Potentiated tumor-specific immunity and its therapeutic limitations. J. Exp. Med. 154:609.

76 Eberlein, T.J., M. Rosenstein and S.A. Rosenberg 1982. Regression of a disseminated syngeneic solid tumor by systemic transfer of lymphoid cells expanded in Interleukin 2. J. Exp. Med., 156:385.

77 Efrat, S., S. Pilo and R. Kaempfer 1982. Kinetics of induction and molecular size of mRNAs encoding human interleukin-2 and gamma-interferon. Nature 297:236.

78 Eggers, A.E., C.A. Hibbard, C.I. Civin, and J.R. Wunderlich 1980. Chemical enhancement of tumor immunogenicity. J. Immunol., 125:1737.

79 Engers, H.D., D. Collavo, M. North, H. von Boehmer, W. Haas, H. Hengartner and M. Nabholz 1980. Characterization of cloned murine cytolytic T cell lines. J. Immunol. 125:1481.

80 Farr, A.G., M.E. Dorf, and E.R. Unanue 1977. Secretion of mediators following T lymphocyte-macrophage interaction is regulated by the major histocompatibility complex. Proc. Natl. Acad. Sci. USA, 74:3542.

81 Farrar, J.J., S.B. Mizel, J. Fuller-Farrar, W.L. Farrar, and M.L. Hilfiker 1980a. Macrophage-independent activation of helper T cells. I. Production of Interleukin 2. J. Immunol. 125:793.

82 Farrar, J.J., J. Fuller-Farrar, P.L. Simon, M.L. Hilfiker, B.M. Stadler and W.L. Farrar 1980b. Thymoma production of T cell growth factor (Interleukin 2). J. Immunol. 125:2555.

83 Farrar, W.L., S.B. Mizel and J.J. Farrar 1980c. Participation of Lymphocyte Activating Factor (Interleukin 1) in the induction of cytotoxic T cell responses. J. Immunol., 124:1371.

84 Farrar, W.L., H.M. Johnson and J.J. Farrar 1981. Regulation of the production of immune interferon and cytotoxic T lymphocytes by Interleukin 2. J. Immunol. 126:1120.

85 Farrar, J.J., W.R. Benjamin, M.L. Hilfiker, M. Howard, W.L. Farrar and J. Fuller-Farrar 1982. The biochemistry, biology and role of Interleukin 2 in the induction of cytotoxic T cells and antibody forming B cell responses. Immunol. Revs. 63:72.

86 Fefer, A., M.A. Cheever and P.D. Greenberg 1982. Overview of prospects and problems of lymphocyte transfer for cancer therapy. Prog. in Cancer Research and Therapy, 22:1.

87 Fernandez-Cruz, E., B. Halliburton and J.D. Feldman 1979. *In vivo* elimination by specific effector cells of an established syngeneic rat maloney virus-induced sarcoma. J. Immunol., 123:1772.

88 Fernandez-Cruz, E., B.A. Woda, and J.D. Feldman 1980. Elimination of syngeneic sarcomas in rats by a subset of T lymphocytes. J. Exp. Med., 152:823.

89 Fernandez-Cruz, E. and J.D. Feldman 1982a. Immunotherapy of progressive tumors with T cell subsets. Prog. in Cancer Res. and Therapy, 22:53.

90 Fernandez-Cruz, E., S.C. Gilman, and J.D. Feldman 1982b. Immunotherapy of a chemically-induced sarcoma in rats: Characterization of the effector T cell subset and nature of suppression. J. Immunol., 128:1112.

91 Finke, J.H., S.D. Sharma, and J.W. Scott 1981. Generation of alloreactive cytotoxic T lymphocytes: Production of T cell and macrophage helper factors in addition ot IL1 and IL2 by peritoneal cells from mice immunized to Listeria Monocytogenes. J. Immunol. 127:2354.

92 Finke, J.H., J. Scott, S. Gillis, and M.L. Hilfiker 1983. Generation of alloreactive cytotoxic T lymphocytes: Evidence for a differentiation factor distinct from IL2. J Immunol. 130:763.

93 Fischer, A., P.C.L. Beverley and M. Feldman 1981. Long-term human T-helper lines producing specific helper factor reactive to influenza virus. Nature 294:166.

94 Fitch, F.W., J.M. Ely, M.B. Prystowsky, D.P. Dialynas, and A.L. Glasebrook 1982. Murine T cell clones having defined immunological functions. Prog. in Cancer Res. and Therapy, 22:229.

95 Fortner, G.W., O. Kuperman and Z.J. Lucas 1975. Immune response to a syngeneic mammary adenocarcinoma. I. Comparison of kinetics of tumor cell growth and cytotoxic responses in syngeneic and allogeneic rats. J. Immunol., 115:1269.

96 Fujimoto, S., T. Matsuzawa, K. Nakagawa, and T. Tada 1978. Cellular interaction between cytotoxic and suppressor T cells against syngeneic tumors in the mouse. Cell. Immunol., 38:378.

97 Fyfe, D.A. and J.H. Finke 1979. Soluble helper factor(s) participates in the

generation of cell mediated cytotoxicity directed against syngeneic tumor cells. J. Immunol., 122:1156.

98 Gerhart, S., G. Mills, V. Monticone, and V. Paetkau 1976. Quantitative analysis of the proliferative activity induced in murine thymocytes by concanavalin A. J. Immunol., 117:1314.

99 Gillis, S. and K.A. Smith 1977a. Long term culture of tumour-specific cytotoxic T cells. Nature, 268:154.

100 Gillis, S., and Smith, K.A. 1977b. In vitro generation of tumor-specific cytotoxic lymphocytes. Secondary allogeneic mixed tumor lymphocyte culture of normal murine spleen cells. J. Exp. Med., 146:468.

101 Gillis, S., M.M. Ferm, W. Ou, and K. Smith 1978a. T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol., 120:2027.

102 Gillis, S., P.E. Baker, F.W. Ruscetti and K.A. Smith 1978b. Long term culture of human antigen-specific cytotoxic T cell lines. J. Exp. Med., 148:1093.

103 Gillis, S., G.R. Crabtree, and K.A. Smith 1979a. Glucocorticoid-induced inhibition of T cell growth factor production. II. The effect on the in vitro generation of cytolytic T cells. J. Immunol., 123:1632.

104 Gillis, S., N.A. Union, P.E. Baker, and K.A. Smith 1979b. The in vitro generation and sustained culture of nude mouse cytolytic T-lymphocytes. J. Exp. Med., 149:1460.

105 Gillis, S. and J. Watson 1980. Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an Interleukin 2-producing human leukemia T cell line. J. Exp. Med. 152:1709.

106 Gillis, S., K.A. Smith and J. Watson 1980. Biochemical characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. J. Immunol. 124:1954.

107 Gillis, S. and S.B. Mizel 1981. T cell lymphoma for the analysis of Interleukin mediated T cell activation. Proc. Natl. Acad. Sci. 78:1133

108 Gillis, S., and C.S. Henney 1981. The biochemical and biological characterization of lymphocyte regulatory molecules. VI. Generation of a B cell hybridoma whose antibody product inhibits IL2 activity. J. Immunol. 126:1978.

109 Gillis, S., A.E. Gillis, and C. Henney 1981. Monoclonal antibody directed against Interleukin 2. I. Inhibition of T lymphocyte mitogenesis and the *in vitro* differentiation of alloreactive cytolytic T cells. J. Exp. Med. 154:983

110 Gillis, S., and J. Watson 1981a. Interleukin 2 dependent culture of cytolytic T cell lines. Immunol. Rev. 54:81.

111 Gillis, S. and J. Watson 1981b. Interleukin 2 induction of hapten-specific cytolytic T cells in nude mice. J. Immunol., 126:1245.

112 Gilman, S.C., J.S. Rosenberg, and J.D. Feldman 1982. T lymphocytes of young and aged rats. II. Functional defects and the role of Interleukin-2. J. Immunol., 128:644.

113 Gilman, S.C., J.S. Rosenberg and J.D. Feldman 1983. Inhibition of Interleukin synthesis and T cell proliferation by a monoclonal anti-Ia antibody. J. Immunol. 130:1236.

114 Glasebrook, A.L. and F.W. Fitch 1979. T cell lines which cooperate in generation of specific cytolytic activity. Nature, 278:171.

115 Glasebrook, A.L. and F.W. Fitch 1980. Alloreactive cloned T cell lines. I. Interactions between cloned amplifier and cytolytic T cell lines. J. Exp. Med. 151:876.

116 Glasebrook, A.L., J. Quintans, L. Eisenberg and F.W. Fitch 1981a. Alloreactive cloned T cell lines. II. Polyclonal stimulation of B cells by a cloned helper T cell line. J. Immunol. 126:240.

117 Glasebrook, A.L., M. Sarmiento, M.R. Loken, D.P. Dialynas, J. Quintans, L. Eisenberg, C.T. Lutz, D. Wilde and F.W. Fitch 1981b. Murine T lymphocyte clones with distinct immunological functions. Immunol. Rev. 54:225.

118 Glaser, M., G.D. Bonnard, R.B. Herberman 1976a. In vitro generation of secondary cell-mediated cytotoxic response against a syngeneic Gross Virus-induced lymphoma in rats. J. Immunol., 116:430.

119 Glaser, M., D.H. Lavrin, and R.B. Herberman 1976b. *In vivo* protection against syngeneic gross virus-induced lymphoma in rats: Comparison with *in vitro* studies of cell-mediated immunity. J. Immunol., 116:1507.

120 Golub,S.H., and D.L. Morton 1974. Sensitisation of lymphocytes *in vitro* against human melanoma-associated antigens. Nature 251:161.

121 Gootenberg, J.E., F.W. Ruscetti, J.W. Mier, A. Gazdar and R.C. Gallo 1981. Human cutaneous T cell lymphoma and leukemia cell lines produce and respond to TCGF. J. Exp. Med. 154:1403.

122 Gorman, R.D. and D.P. Fan 1983. Characterization of helper factors distinct from interleukin 2 necessary for the generation of allospecific cytolytic T lymphocytes. J. Immunol. 130:756.

123 Green, D.R., 1982a. Contrasuppression: Its Role in Immunoregulation. Prog. in Cancer Res. Therapy, 22:279.

124 Green, D.R., 1982b. The *in vitro* generation of H-2-restricted cytotoxic T cells to AKR/Gross leukemia virus-induced tumors 1. Requirements for stimulation with allogeneic leukemia cells *in vivo*. J. Immuno., 128:1043.

125 Greenberg, P.D., M. Cheever, and A. Fefer 1979. Suppression of the *in vitro* secondary response to syngeneic tumor and of *in vivo* tumor therapy with immune cells by culture-induced suppressor cells. J. Immunol. 123:515.

126 Greenberg, P.D., M.A. Cheever, and A. Fefer 1980. Detection of early and delayed antitumor effects following curative adoptive chemoimmunotherapy of established leukemia. Cancer Research 40:4428.

127 Greenberg, P.D., M.A. Cheever, and A. Fefer 1981a. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1+2- Lymphocytes. J. Exp. Med. 154:952.

128 Greenberg, P.D., M.A. Cheever and A. Fefer 1981b. H-2 restriction of adoptive immunotherapy of advanced tumors. J. Immunol., 126:2100.

129 Greenberg, P.D., M.A. Cheever and A. Fefer 1982. Prerequisites for successful adoptive immunotherapy: Nature of effector cells and role of H-2 restriction. Prog. in Cancer Res. and Therapy, 22:31.

130 Gullberg, M., F. Ivars, A. Coutinho and E.-L. Larsson 1981. Regulation of T cell

growth factor production: Arrest of TCGF production after 18 hours in normal lectin-stimulated mouse spleen cell cultures. J. Immunol. 127:407.

131 Gullberg, M. and E.-L. Larsson 1982. Studies on induction and effector functions of Con A-induced suppressor cells that limit TCGF production. J. Immunol. 128:746.

132 Haagensen, Jr., D.E., G. Rolson, J.J. Colins, S.A. Wells, Jr., D.P. Bolognesi, and H.J. Hansen 1978. Immunologic control of the ascites form of murine adenocarcinoma 755. I. Protection with syngeneic immune serum or lymphoid cells. J. Natl. Cancer Inst., 60:131.

133 Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai 1983. Natural killer cells as a responder to Interleukin 2 (IL2). II. IL2-induced Interferon production. J. Immunol. 130:988.

134 Hapel, A.J., J.C. Lee, W.L. Farrar and J.N. Ihle 1981. Establishment of continuous cultures of Thy1.2+, Lyt1+,2- T cells with purified Interleukin 3. Cell 25:179.

135 Hardt, C., M. Rollinghoff, K. Pfizenmaier, H. Mosmann, and H. Wagner 1981. Ly $1^{-}2^{+}3^{+}$ Cyclophosphamide-sensitive T cells regulate the activity of an Interleukin 2 inhibitor *in vivo*. J. Exp. Med. 154:262.

136 Harwell, L., B. Skidmore, P. Marrack and J. Kappler 1980. Concanavalin A-inducible, Interleukin-2-producing T cell hybridoma. J. Exp. Med. 152:893.

137 Hefeneider, S.H., P.J. Conlon, C.S. Henney, and S. Gillis 1983. In vivo Interleukin 2 administration augments the generation of alloreactive cytolytic T lymphocytes and resident natural killer cells. J. Immunol. 130:322.

138 Hellstrom, K.E., and I. Hellstrom 1974. Lymphocyte mediated cytotoxicity and blocking serum activity to tumor antigens. Advances in Immunology 18:209.

139 Hellstrom, K.E., I. Hellstrom, J.A. Kant, and J.D. Tamerius 1978. Regression and inhibition of sarcoma growth by interference with a radiosensitive T-cell population. J. Exp. Med., 799

140 Henney, C.S., M. Okada and S. Gillis 1980. The cellular and antigenic requirements for IL-2 production *in vitro*. Behring Inst. Mitt., 67:26.

141 Hess, A.D., P.J. Tutschka, and G.W. Santos 1982. Effect of cyclosporin on human lymphocyte responses *in vitro*. III. CsA inhibits the production of T lymphocyte growth factors in secondary mixed lymphocyte responses but does not inhibit the response of primed lymphocytes to TCGF. J. Immunol., 128:335.

142 Herberman, R.B. and J.R. Ortaldo 1981. Natural killer cells: Their role in defences against disease. Science 214:24.

143 Herrman, S.H., O. Weinberger, S.J. Burakoff and M.F. Mescher 1982. Analysis of the two-signal requirement for precursor cytolytic T lymphocyte activation using H-2K in liposomes. J. Immunol., 128:1968.

144 Heuer, J., K. Brunner, B. Opalka and E. Koelsch 1982. A cloned T-cell line from a tolerant mouse represents a novel antigen-specific suppressor cell type. Nature 296:456.

145 Hilfiker, M.L., R.N. Moore and J.J. Farrar 1981. Biologic properties of chromatographically separated murine thymoma-derived interleukin 2 and colony-stimulating factor. J. Immunol. 127:1983.

146 Hofer, K.G., and W.L. Hughes 1971. Radiotoxicity of intranuclear Tritium, ^{125} Iodine and ^{131} Iodine. Rad.Res. 47:94.

147 Howell, S.B., J.H. Dean, E.C. Esber, and L.W. Law 1974. Cell interactions in adoptive immune rejection of a syngeneic tumor. Int. J. Cancer, 14:662.

148 Huber, S., and Z. J. Lucas 1979. A quantitative assay for tumor antigen based on inhibition of cytotoxicity of sensitized lymphocytes. Int. J. Cancer, 23:224.

149 Humphrey, L.J., P.M. Lincoln, and W.D. Griffin Jr. 1968. Immunologic response in patients with disseminated cancer. Ann. Surg., 168:374.

150 Humphrey, L.J., W.R. Jewel, D.R. Murray and W.D. Griffin Jr. 1971. Immunotherapy for the patient with cancer. Ann. Surg., 173:47.

151 Igarashi, T., D. Rodrigues and C. Ting 1979. Studies of the mechanism for the induction of *in vivo* tumor immunity. I.V. Enhancement of the *in vitro* generation of secondary cell-mediated cytotoxic response by normal peritoneal macrophages and their culture supernatants. J. Immunol., 122:1519.

152 Ihle, J.N., L. Pepersack and L. Rebar 1981a. Regulation of T cell differentiation: in vitro induction of 20 alpha-hydroxysteroid dehydrogenase in splenic lymphocytes from athymic mice by a unique lymphokine. J. Immunol. 126:2184.

153 Ihle, J.N., L. Rebar, J. Keller, J.C. Lee and A. Hapel 1981b. Interleukin 3: Possible roles in the regulation of lymphocyte differentiation and growth. Immunol. Revs. 63:101.

154 Ihle, J.N., A. Hapel, J. Greenberger, J.C. Lee, and A. Rein 1982a. Possible roles of Interleukin 3 in the regulation of lymphocyte differentiation. Prog. in Cancer Res. and Therapy, 22:93.

155 Ihle, J.N., J. Keller, L. Henderson, F. Klein, and E. Palaszynski 1982b. Procedures for the purification of Interleukin 3 to homogeneity. J. Immunol. 129:2431.

156 Julius, M.H., E. Simpsom, and L.A. Herzenberg 1973. Arapid method for the isolation of junctional thymus derived cells. Eur. J. Immunol. 3:64.

157 Kaerre, K., G.O. Klein, R. Kiessling, G. Klein and J.C. Roder 1980. Low natural in vivo resistance to syngeneic leukaemias in natural killer deficient mice. Nature 284:624.

158 Kamao, I., and H. Friedman 1977. Immunosuppression and the role of suppressive factors in cancer. Advances in Cancer Research, 25:271.

159 Kantmann, S.H.E., And H. Hahn 1982. Biological functions of T cell lines with specificity for the intracellular bacterium Listeria Monocytogenes *in vitro* and *in vivo*. J. Exp. Med., 1754.

160 Katz, D.H., 1977. Lymphocyte differentiation, recognition and regulation. Academic Press New York.

161 Kedar, E., M. Schwartzbach, Z. Raana and S. Hefetz 1977. *in vitro* induction of cell mediated immunity to murine leukemia cells. II. Cytotoxic activity *in vitro* and tumor neutralizing capacity *in vivo* of anti-leukemic cytotoxic lymphocytes generated in macrocultures. J. Immuno. Methods, 16:39.

162 Kedar,E. and T. Lupul 1978. *In vitro* induction of cell mediated immunity to murine leukemia cells. I.V. Amplification of the generation of cytotoxic lymphocytes

by enzymatically and chemically modified stimulator leukemia cells. J. Immunol. Methods, 21:35.

163 Kedar, E., R.B. Heberman, E. Gorelik, B. Sredni, G.D. Bonnard, and N. Navarro 1982. Antitumor reactivity *in vitro* and *in vivo* of mouse and human lymphoid cells cultured with T cell growth factor. Prog. in Cancer Res. and Therapy, 22:173.

164 Keene, J., and J. Forman 1982. Helper activity is required for the *in vivo* generation of cytotoxic T lymphocytes. J. Exp. Med., 155:768.

165 Keller, D.M., J.E. Swierkosz, P. Marrack and J.W. Kappler 1980. Two types of functionally distinct, synergizing helper T cells. J. Immunol., 123:1350.

166 Kern, D.E., S. Gillis, M. Okada and C. Henney 1981. The role of Interleukin 2 (IL2) in the differentiation of cytotoxic T cells: The effect of monoclonal Anti-IL2 antibody and absorption with IL2 dependent T cell lines. J. Immunol. 127:1323.

167 Kilburn, D.G., F.O. Talbot, H.-S. Teh and J.G. Levy 1979. A specific helper factor which enhances the cytotoxic response to a syngeneic tumour. Nature 277:474.

168 Klein, J.R., D.H. Raulet, M.S. Pasternack and M.J. Bevan 1982. Cytotoxic T lymphocytes produce immune interferon in response to antigen or mitogen. J. Exp. Med. 155:1198.

169 Kornbluth, J., D.M. Silver and B. Dupont 1981. Cloning and characterization of primary alloreactive human T lymphocytes. Immuno. Rev. 54:111.

170 Kramer, M., and U. Koszinowski 1982. T cell-specific suppressor factor(s) with regulatory influence on Interleukin 2 production and function. J. Immunol. 128:784.

171 Kripke, M.L., M.B. Bundmen and I.J. Fidler 1977. Production of specific macrophage activating factor by lymphocytes from tumor-bearing mice. Cell Immunol. 30:341.

172 Kung, P., G. Goldstein, E.L. Reinherz and S. Schlossman 1979. Monoclonal antibodies defining distinctive T cell surface antigens. Science 206: 347.

173 Kuperman, O., G.W. Fortner and Z.J. Lucas 1975a. Immune response to a syngeneic mammary adenocarcinoma. II. In vitro generation of cytotoxic lymphocytes. J. Immunol., 115:1277.

174 Kuperman, O., G.W. Fortner and Z.J. Lucas 1975b. Immune response to a syngeneic mammary adenocarcinoma. III. Development of memory and suppressor functions modulating cellular cytotoxicity. J. Immunol., 115:1282.

175 Kuribayashi, K., S. Gillis, D. Kerr and C. Henney 1981. Murine N.K. cell cultures: Effects of Interleukin 2 and Interferon on cell growth and cytotoxic reactivity. J. Immunol. 126:2321.

176 Lafferty, K.J., H.S. Warren, J.A. Woolnough, and D.W. Talmage 1978. Immunological induction of T lymphocytes: Role of antigen and the lymphocyte costimulator. Blood Cells, 4:395.

177 Lalande, M., M.J. McCutcheon and R.G. Miller 1980. Quantitative studies on the precursors of cytotoxic lymphocytes. VI. Second signal requirements of specifically activated precursors isolated 12 hours after stimulation. J. Exp. Med. 151:12.

178 Larsson, E.-L., 1980. Cyclosporan A and dexamethasone suppresses T cell responses by selectively acting at distinct sites of the triggering process. J. Immunol., 124:2828.

179 Larsson, E.-L., and A. Coutinho 1980. Mechanism of T cell activation. I. A screening of step one ligands. Eur. J. Immunol. 10:93.

180 Larsson, E.-L., N.N. Iscove and A. Coutinho 1980a. Two distinct factors are required for induction of T cell growth. Nature, 283:664.

181 Larsson, E.-L, A. Coutinho, and C. Mortinez 1980b. A suggested mechanism for T lymphocyte activation: Implications on the acquisition of functional reactivities. Immunol. Rev., 51:61.

182 Larsson, E.-L, 1981. Mechanism of T cell activation antigen and lectin-dependent acquisition of responsiveness to TCGF is a mon-mitogenic, active response of resting T cells. J. Immunol., 126:1323.

183 Larsson, E.-L. 1982. Functional heterogeneity of helper T cells: Two distinct helper T cell are required for the production of T cell growth factor. J. Immunol. 128:742.

184 Lattime, E.C., S. Gillis, G. Pecoraro, and O. Stuttman 1982. Ia-dependent Interleukin 2 production in syngeneic cellular interactions. J. Immunol., 128:480.

185 Lee, S.K., and R.T.D. Oliver 1978. Autologous leukemia-specific T-cell-mediated lymphocytoxicity in patients with acute mylogenous leukemia. J. Exp. Med. 147:912.

186 Levy, J.G., A.G. Smith, R.B. Whitney, R. McMaster and D.G. Kilburn 1976. Characterization of a T-lymphocyte inhibitor in the serum of tumor-bearing mice. J. Immunol. 30:565.

187 Levy, J.P., and J.C. Leclerc 1977. The murine sarcoma virus-induced tumor: Exception or general model in tumor immunology. Adv. in Cancer Res., 24:1.

188 Lin, Y., B.M. Stadler and H. Rabin 1982. Synthesis of biologically active Interleukin 2 by Xenopus oocytes. J. Biol. Chem. 257:1587.

189 Lopez-Botet, M., G. Foutan, M.C.G. Rodriquez and M.O. de Landazuri 1982. Relationship between IL2 synthesis and the proliferative response to PHA in different primary immunodefficiencies. J. Immunol., 128:679.

190 Lotze, M.T., B.R. Line, D.J. Mathisen and S.A. Rosenberg 1980. The in vivo distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): Implications for the adoptive immunotherapy of tumors. J. Immunol. 125:1487.

191 Loveland, B.E., P.M. Hogarth, R.H. Ceridig and I.F.C. Mackenzie 1981. Cells mediating graft rejection in the mouse 1. Lyt-1 cells mediate skin graft rejection. J. Exp. Med., 153:1044.

192 Loveland, B.E. and I.F.C. McKenzie 1982. Cells mediating graft rejection in the mouse. II. The LY phenotypes of cells producing tumor allograft rejection. Transplantation 33:174.

193 Lukasewycz, O.A., P.S. Duffey, and W.H. Murphy 1976. Immune mechanisms in leukemia: Protective capacity of the major lymphoid cell compartments. J. Immunol. 116:976.

194 Lundak, R.L., and D.J. Raidt 1973. Cellular immune response against tumor cells. I.

In vitro immunization of allogeneic and syngeneic mouse spleen cell suspensions against DBA mastocytoma cells. Cell. Immun. 9:60.

195 MacDonald, H.R., J.C. Cerottini, J.E. Ryser, J.L. Maryanski, C. Taswell, M.B. Widmer and K.T. Brunner 1980. Quantitation and cloning of cytolytic T lymphocytes and their precursors. Immunol. Rev., 5l:93.

196 McKimm-Breschkin, J.L., P.L. Muttram, W.R. Thomas and J.F.A.P. Miller 1982. Antigen specific production of Immune Interferon by T cell lines. J. Exp. Med. 155:1204.

197 Meltzer, M. and J.J. Oppenheim 1977. Bidirectional amplification of macrophage-lymphocyte interactions:Enhanced lymphocyte activation factor production by activated mouse peritoneal cells. J. Immunol., ll8:77

198 Merluzzi, V.J., M.M. Walker and R.B. Faanas 1981a. Inhibition of cytotoxic T-cell clonal expansion by cyclophosphamide: Recovery of CTL precursors by supernatants from mixed lymphocyte cultures. Cancer Res., 41:850.

199 Merluzzi, V.J., R.E. Kenney, F.A. Schnid, Y.S. Choi and R.B. Faanes l981b. Recovery of the *in vivo* cytotoxic T-cell response in Cyclophosphamide - treated mice by injection of mixed - lymphocyte - culture supernatants. Cancer Res., 41:3663.

200 Mier, J.W. and R.C. Gallo 1980. Purification and some characteristics of human T-cell growth factor from phytohemagglutinin-stimulated lymphocyte-conditioned media. Proc. Natl. Acad. Sci. USA 77:6134.

201 Miller, R.G. and M. Dunkley 1974. Quantitative analysis of the chromium release cytotoxicity assay for cytotoxic lymphocytes. Cell. Immunol., 14:284.

202 Miller, R.G., M.E. LaLande, H. Derry and V. Paetkau 1980. Second signal requirements of cytotoxic T lymphocyte precursors. Behring Inst. Mitt. 67:41.

203 Miller, R.A., and O. Stuttman 1982. Enumeration of IL2-secreting helper T cells by limiting dilution analysis, and demonstration of unexpectedly high levels of IL2 production per responding cell. J. Immunol., 128:2258.

204 Mills, C.D., R.J. North and E.S. Dye 1981. Mechanisms of anti-tumor action of C. parvum. II. Potentiated cytolytic T cell response and its tumor-induced suppression. J. Exp. Med. 154:621.

205 Mills, G., V. Monticone, and V. Paetkau 1976. The role of macrophages in thymocyte mitogenesis. J. Immunol., 117:1325.

206 Mills, G.B. and V. Paetkau 1980. Generation of cytotoxic lymphocytes to syngeneic tumor by using costimulator (Interleukin 2). J. Immunol. 125:1897.

207 Mills, G.B., G. Carlson and V. Paetkau 1980. Generation of cytotoxic lymphocytes to syngeneic tumors using costimulator (IL2): In vivo activity. J. Immunol. 125:1904.

208 Mitchison, N.A., 1955. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. J. Exp. Med., 102:57.

209 Mitchison, N.A., 1971. The carrier effect in the secondary response to hapten protein conjugates. II. Cellular cooperation. Eur. J. Immunol., 1:18.

210 Miyawaki, T., A. Yachie, N.S. Uwadana, S. Ohzeki, T. Nagaoki, and N. Taniguchi 1982. Functional significance of Tac antigen expressed on activated human T

lymphocytes. Tac antigen interacts with T cell growth factor in cellular proliferation. J. Immunol. 129:2474.

211 Mizel, S.B., J.J. Oppenheim, and D.L. Rosenstreich 1978a. Characterization of lymphocyte-activating factor (LAF) produced by the macrophage cell line, P388D1. I. Enhancement of LAF production by activated T lymphocytes. J. Immunol., 120:1497.

212 Mizel, S.B., D.L. Rosenstreich, and J.J. Oppenheim 1978b. Phorbol myristic acetate stimulates LAF production by the macrophage cell line P388D1. Cell. Immunol., 40:230.

213 Mizel, S.B. 1979. Physiochemical characterization of lymphocyte-activating factor (LAF). J. Immunol., 122:2167.

214 Mizel, S.B. and D. Mizel 1981. Purification to apparent homogeneity of murine interleukin 1. J. Immunol. 126:834.

215 Morgan, D.A., F. W. Ruscetti, and R. Gallo 1976. Selective *in vitro* growth of T lymphocytes from normal human bone marrow. Science 193:1007.

216 Moore, R.N., J.J. Oppenheim, J.J. Farrar, C.C. Carter, A. Waheed, and T. Shadduck 1980. Production of Lymphocyte-Activating Factor (Interleukin 1) by macrophages activated with Colony Stimulating Factors. J. Immunol., 125:1302.

217 Morris, A.G., Y.L. Lin and B.A. Askonas 1982. Immune interferon release when a cloned cytotoxic T-cell line meets its correct-influenza-infected target cell. Nature 295:150.

218 Nadler, S.H., and G.E. Moore 1969. Immunotherapy of Malignant disease. Archs. Surg. 99:376.

219 Naor, D., 1979. Suppressor cells: Permitters and promoters of malignancy. Adv. Cancer Res. 29:45.

220 Naparstek, Y., J. Holoshitz, S. Eisenstein, T. Reshef, S. Rappaport, J. Chemke, A. Ben-Nun, and I.R. Cohen 1982. Effector T lymphocyte line cells migrate to the thymus and persist there. Nature 300:262.

221 Neefe, J.R., J.E. Sullivan, and R.J. Hartzman 1982. Clonal analysis of restriction antigens for human TNP-specific cytotoxic T lymphocytes. J. Immunol. 128:227.

222 North, R.J. 1982. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J. Exp. Med., 55:1063.

223 North, R.J., E.S. Dye, and C.D. Mills 1982. T cell mediated negative regulation of concomitant antitumor immunity as an obstacle to adoptive immunotherapy of established tumors. Prog. in Cancer Res. and Therapy, 22:65.

224 Northoff, H., C. Carter and J.J. Oppenheim 1980a. Inhibition of concanavalin A-induced human lymphocyte mitogenic factor (Interleukin-2) production by suppressor T lymphocytes. J. Immunol. 125:1823.

225 Northoff, H., J.J. Hooks, G. Jordan and J.J. Oppenheim 1980b. Direct correlation between the production of Interleukin 2 (IL-2) and Immune Interferon (IF) by human mononuclear cells (MNL). Behring Inst. Mitt., 67:90.

226 Okada, M., G.R. Klimpel, R.C. Kuppers, and C.S. Henney 1979. The differentiation of cytotoxic T cells in vitro. I. Amplifying factor(s) in the

primary response is Lyt 1+ cell dependent. J. Immunol., 122:2527.

227 Okada, M., T.H. Stanton, R.C. Kuppers, and C.S. Henney 1981. The differentiation of cytotoxic cells *in vitro*. IV. Interleukin 2 production in primary mixed lymphocytes involves cooperation between Qa-1+ and Qa-1- helper T cells. J. Immunol., 126:1635.

228 Oppenheim, J.J., S.B. Mizel and M.S. Meltzer 1978. Comparison of lymphocyte and mononuclear phagocyte derived "amplification" factors. *In* Biology of the lymphokines. Edited by S. Cohen, E. Pick and J.J. Oppenheim. Academic Press New York.

229 Ortaldo, J.R., T.T. Timonen, B.M. Bose, and J.A. Alvarez 1982. Human natural killer cells as well as T cells maintained in continous cultures with IL-2. Prog. in Cancer Res. and Therapy, 22:191.

230 Osawa, H., and T. Diamantstein 1983. The characteristics of a monoclonal antibody that binds specifically to rat T lymphoblasts and inhibits IL2 receptor functions. J. Immunol. 130:51.

231 Paciucci, P.A., S. Macphail, J.M. Zarling and F.H. Bach 1980. Lysis of syngeneic solid tumor cells by alloantigen stimulated mouse T and non-T cells. J. Immunol., 124:370.

232 Paetkau, V., G. Mills, S. Gerhart, and V. Monticone 1976. Proliferation of murine thymic lymphocytes in vitro is mediated by the concanavalin A-induced release of a lymphokine (costimulator). J. Immunol. 117:1320.

233 Paetkau, V., J. Shaw, B. Caplan, G.B. Mills and K.-C. Lee 1980a. Interleukin 2 in cell-mediated immune responses. J. Supramol. Structure 13:271.

234 Paetkau, V., J. Shaw, G. Mills and B. Caplan 1980b. Cellular origins and targets of costimulator (IL2). Immunol. Revs. 51:157.

235 Paetkau, V. 1981. Thymocyte stimulating factors. In: The Lymphokines, Ed. by J. Hadden and W.E. Stewart II, Humana Press, Clifton, New Jersey. pp 131.

236 Paetkau, V., G.B. Mills, and R.C. Bleackley 1982. Enhancement of anti-tumor immune responses with Interleukin 2. Prog. in Cancer Res. and Therapy, 22:147.

237 Palacios, R., and G. Moller 1981. T cell growth factor abrogates conconavalin A - induced suppressor cell function. J. Exp. Med., 153:1360.

238 Palacios, R., and O. Martinez-Maza 1982. Is the E receptor on human T lymphocytes a "negative signal receptor"? J. Immunol. 129:2479.

239 Palacios, R. 1982a. Cloned lines of Interleukin 2 producer human T lymphocytes. J. Immunol. 129:2586.

240 Palacios, R. 1982b. Mechanism of T cell activation. Role and functional relationship of HLA-DR antigens and interleukins. Immunol. Rev. 63:73.

241 Pfizenmaier, K. 1982. Interleukin-producing T cell hybridomas: A biological characterization of mediators controlling lymphocyte activation. In: Lymphokines Vol. 5, Monoclonal T Cells and Their Products, Ed. M. Feldmann and M.H. Schreier, Academic Press, pp 323.

242 Pilarski, L.M., P.A. Bretscher, and L.L. Baum 1977. Helper T cells are required or the polyclonal stimulation of cytotoxic T cells by concanavalin A. J. Exp. Med.. 145:1237.

243 Pilarski, L.M. 1977. A requirement for antigen-specific helper T cells in the generation of cytotoxic T cells from thymocyte precursors. J. Exp. Med., 145:709.

244 Pilarski, L.M. 1979. Antigen-specific helper T cells are essential for cytotoxic T cell responses to metabolically inactivated stimulator cells. Eur. J. Immunol., 9:454.

245 Plata, F. and C.V. Jongeneel 1977. Characteriztion of effector lymphocytes associated with immunity to murine sarcoma virus (MSV)-induced tumors. II. Repeated stimulation and proliferation in vitro of specific cytolytic T lymphocytes. J. Immunol., 119:623.

246 Plate, J.M.D. 1977. Two signals required for the initiation of killer T-cell differentiation. Cell. Immunol., 32:183.

247 Pollack, S.B., M.R. Tam, R.C. Nowinski and S.L. Emmons 1979. Presence of T cell-associated surface antigens on murine NK cells. J. Immunol., 123:1818.

248 Portens,D.D. and T.R. Munro 1972. The kinetics of the killing of mouse tumor cells *in vivo* by immune responses. Int. J. Cancer 10:112.

249 Postlethwaite, A.E., L.B. Lachman, C.L. Mainardi, and A.H. Kang 1983. Interleukin 1 stimulation of collagenase produciton by cultured fibroblasts. J. Exp. Med. 157:801.

250 Poupon, M.F., B. Payelle and G. Lespinats 1981. Adoption of tumor immunity: Role of the major histocompatibility complex. J. Immunol., 126:2342.

251 Prystowsky, M.B., J.M. Ely, D.I. Beller, L. Eisenberg, J. Goldman, M. Goldman E. Goldwasser, J. Ihle, J. Quintans, H. Remold, S.N. Vogel and F.W. Fitch 1982. Alloreactie cloned T cell lines. VI. Multiple lymphokine activities secreted by helper and cytolytic cloned T lymphocytes. J. Immunol. 129:2337.

252 Putman, D.L., P.D. Kind, A. Goldin and M. Kende 1978. Adoptive immunochemotherapy of a transplantable AKR leukemia (K36). Int. J. Cancer 21:230.

253 Rabin, H., R.F. Hopkins, F.W. Ruscetti, R.H. Neubauer, R.L. Brown, and T.G. Kawanami 1981. Spontaneous release of a factor with properties of T cell growth factor from a continuous line of primate tumor-T-cells. J. Immunol., 127:1852.

254 Rao, A., S.B. Mizel, and H. Cantor 1983. Disparate functional properties of two interleukin 2 responsive Ly 1^+ 2^- T cell clones: Distinction of T cell growth factor and T cell-replacing factor activities. J. Immunol. 130:1743.

255 Rappaport, R., and G. R. Dodge 1982. Prostaglandin E. inhibits the production of human Interleukin 2. J. Exp. Med., 155:943.

256 Raulet, D.H., and M.J. Bevan 1982. A differentiation factor required for the expression of cytotoxic T-cell function. Nature 296:754.

257 Reddehase, M., W. Suessmuth, C. Moyers, W. Falk, and W. Droege 1982. Interleukin 2 is not sufficient as helper component for the activation of cytotoxic T lymphocytes but synergizes with a late helper effect that is provided by irradiated I-region-incompatible stimulator cells. J. Immunol., 128:61.

258 Robb, R.J. and K.A. Smith 1981. Heterogeneity of human TCGF due to variable glycosylation. Mol. Immunol. 18:1087.

259 Robb, R.J., A. Munck and K.A. Smith 1981. TCGF receptors. Quantitation, specificity and biological relevance. J. Exp. Med. 154:1455.

260 Roberts, L.K., C. W. Spellman, J.V. Giorgi, A. Tai, and N.L. Warner 1982. Preliminary characterization of a series of tumor reactive continuous cell lines. Prog. in Cancer Res. and Therapy, 22:207.

261 Rock, K.L. 1982. The role of Ia molecules in the activation of T lymphocytes. 1. The activation an IL 1-dependent Il2 producing T cell hybridoma by Con A requires and interaction, which is not H-2 restricted, with an Ia bearing accessory cell. J. Immunol. 129:1360.

262 Roder, J.C., T. Haliotis, M. Klein, S. Korec, J.R. Jeff, J. Ortaldo, R.B. Heberman, P. Katz and A.S. Fauci 1980. A new immunodeficiency disorder in humans involving N.K. cells. Nature 284:553.

263 Roder, J.C., and T. Haliotis 1980. Do NK cells play a role in anti tumor surveillance? Immunology Today, 1980:96.

264 Roder, J.C., K. Karre and R. Kiessling 1981. Natural Killer Cells. Progress in Allergy 28:66.

265 Rollinghoff, M., and H. Wagner 1973. *In vitro* induction of tumor specific immunity: Requirements for T lymphocytes and tumor growth inhibition *in vivo*. Eur. J. Immunol., 3:471.

266 Rollinghoff, M., A. Starzinski-Powitz, K. Pfizenmaier and H. Wagner 1977. Cyclophosphamide - sensitive T lymphocytes suppress the *in vivo* generation of antigen-specific cytotoxic T lymphocytes. J. Exp. Med., 145:455.

267 Rosenberg, S.A., and W.D. Terry 1977. Passive immunotherapy in animals and man. Advances in Cancer Research 25:323.

268 Rosenberg, S.A., T. Eberlein, E. Grimm, A. Mazumder, and M. Rosenstein 1982. Adoptive transfer of lymphoid cells expanded in T-Cell Growth Factor: Murine and human studies. Prog. in Cancer Res. and Therapy, 22:161.

269 Rosenberg, J.S., S.C. Gilman, and J.D. Feldman 1983. Effects of aging on cell cooperation and responsiveness to cytokines. J. Immunol. 130:1754.

270 Rosenstreich, D.L., J.J. Farrar and S. Dougherty 1976. Absolute macrophage dependency of T lymphocyte activation by mitogens. J. Immunol., 116:131.

271 Rosenthal, A.S., M.A. Barcinski, and L.J. Rosenwasser 1978. Function of macrophages in genetic control of immune responsiveness. Fed. Proc., 37:79.

272 Rosenthal, A.S., 1980. Regulation of the immune response-Role of the macrophage. N. Engl. J. Med., 303:1153.

273 Rouse, B.T., M. Rollinghoff and N.L. Warner 1973. Tumor immunity to murine plasma cell tumors. II. Essential role of T lymphocytes in immune response. Eur. J. Immunol., 3:218.

274 Rulon, K. and D.W. Talmage 1979. Enhancement of the primary cytotoxic response to membrane by a lymphokine costimulator. Proc. Natl. Acad. Sci. USA, 76:1994.

275 Ruscetti, F.W., D. Morgan, and R.C. Gallo 1977. Functional and morpholigic characterization of human T cells continuously grown *in vitro*. J. Immunol. 119:131.

276 Ryser, J.-E., Cerottini, J.-C., and Brunner, K.T. 1979. Cell mediated immunity to antigens associated with murine sarcoma virus induced tumors: Augmentation of cytolytic T lymphocyte activity by successive specific and nonspecific stimulation in vitro. Eur. J. Immunol., 9:179.

277 Sagamura, K., Y. Tanaka and Y. Hinuma 1982. Two distinct human cloned T cell lines that exhibit natural killer-like and anti-human effector activities. J. Immunol. 128:1749.

278 Schaaf-Lafontaine, N., 1978. Separation of Lymphoid cells with a suppressor effect on the activity of cytotoxic cells *in vitro* during the growth of a syngeneic mouse tumor. Int. J. Cancer, 21:329.

279 Schirrmacher, V., 1979. Tumor metastases and cell-mediated immunity in a model system in DBA/2 mice. V. Transfer of protective immunity with H-2 identical immune T cells from B10.D2 mice. Int. J. Cancer, 24:80.

280 Schreier, R.D., B.J. Skidmore, J.T. Kurnick, S.N. Goldstein, and J.M. Chiller 1979. Propagation of antigen specific T cell helper function in vitro. J. Immunol., 123:2525.

281 Schreier, M.H., N. Iscore, R. Tees, L. Aarden and H. Von Boehmer 1980. Clones of killer and helper T cells: Growth requirements, specificity and retention of function in long term culture. Immunol. Rev. 51:315.

282 Schreier, M.H., and R. Tees 1980. Clonal induction of helper T cells: Conversion of specific signals into non-specific signals. Int. Archs. Allergy Appl. Immunol. 61:227.

283 Shaw, J., V. Monticone, G. Mills, and V. Paetkau 1978a. Effects of costimulator on immune responses in vitro. J. Immunol., 120:1974.

284 Shaw, J., V. Monticone, and V. Paetkau 1978b. Partial purification and molecular characterization of a lymphokine (costimulator) required for the mitogenic response of mouse thymocytes in vitro. J. Immunol., 120:1967.

285 Shaw, J., B. Caplan, V. Paetkau, L.M. Pilarski, T.L. Delovitch and I.F.C. McKenzie 1980. Cellular origins of costimulator (IL2) and its activity in cytotoxic T lymphocyte responses. J. Immunol. 124:2231.

286 Sheehy, M.J., F.B. Quintieri, D.Y.M. Leung, R.S. Geha, D.P. Dubey, C.E. Limmer, and E.J. Yunis 1983. A human large granular lymphocyte clone with natural killer-like activity and T cell-like markers. J. Immunol. 130:228.

287 Shu, S., J.T. Hunter, H.J. Rapp, and L.S. Fonseca 1982. Adoptive immunity to a syngeneic guinea pig hepatoma: Characteristics of effectors and quantitative analysis of tumor rejection. Prog. in Cancer Res. and Therapy, 22:79.

288 Simon, P.L., J.J. Farrar, and P.D. Kind 1979. Biochemical relationship between murine immune interferon and a killer cell helper factor. J. Immunol., 122:127.

289 Small, M., and N. Trainin 1975. Inhibition of syngeneic fibrosarcoma growth by lymphocytes sensitized on tumor-cell monolayers in the presence of the thymic humoral factor. Int. J. Cancer, 15:962.

290 Smith, H.G., R.P. Harmel, M.G. Hanna, Jr., B.S. Zwilling, B. Zbar, and H.J. Rapp 1977. Regression of established intradermal tumors and lymph node metastases in guinea pigs after systemic transfer of immune lymphoid cells. J. Nat. Cancer Inst., 58:1315.

291 Smith, K.A. 1980. Continuous cytotoxic T-cell lines. Contemporary Topics in

Immunobiology 11:139.

292 Smith, K.A., Gilbride, K.J. and Favata, M.F. 1980a. Lymphocyte activating factor promotes T-cell growth factor production by cloned murine lymphoma cells. Nature 287:853.

293 Smith, K.A., K.J. Gilbride and M.F. Favata 1980b. Interleukin 1 - promoted Interleukin 2 production. Behring Inst. Mitt., 67:4.

294 Smith, K:A., L.B. Lachman, J.J. Oppenheim and M.F. Favata 1980c. The function relationship of the interleukins. J. Exp. Med. 151:1551.

295 Snyder, D.S., and E.R. Unanue 1982. Corticosteroids inhibit murine macrophage Ia expression and interleukin 1 production. J. Immunol. 129:1803.

296 Solbach, W., S. Barth, M. Granbuer, M. Rollinghoff and H. Wagner 1980. Sezary Syndrome: Evidence for malignant proliferation of T cells producing Interleukin 2. Behring Inst. Mitt., 67:265.

297 Spits, H., J. Borst, C. Terhorst, and J.E. deVries 1983. The role of T cell differentiation markers in antigen-specific and lectin-dependent cellular cytotoxocity mediated ty $T8^+$ and $T4^+$ human cytotoxic T cell clones directed at class I and class II MHC antigens. J. Immunol. 129:1563.

298 Sredni, B., H.Y Tse and R.H. Schwartz 1980. Direct cloning and extended culture of antigen specific MHC-restricted, proliferating T lymphocytes. Nature 283:581.

299 Sredni, B. and R.H. Schwartz 1980. Alloreactivity of an antigen-specific T-cell clone. Nature 287:855.

300 Sredni, B., and R.H. Schwartz 1981. Antigen-specific, proliferating T lymphocyte clones. Methodology, specificity, MHC restriction and alloreactivity. Immunol. Rev. 54:185.

301 Sredni, B., H.Y. Tse, C. Chen and R.H. Schwartz 1981. Antigen-specific clones of proliferating T lymphocytes. I. Methodology, specificity, and MHC restriction. J. Immunol. 126:341.

302 Suzuki, R., K. Handa, K. Itoh, and K. Kumagai 1983. Natural killer cells as a responder to Interleukin 2 (IL2). I. Proliferative response and establishment of cloned cells. J. Immunol. 130:981.

303 Swain, S., 1980. Role of IA positive cells in the production of T cell replacing factors: Blocking of factor production with Anti-IA serum. J. Immunol., 125:1224.

304 Swain, S.L., and R.W. Dutton 1980. Production of Con A-induced helper T cell replacing factor requires a T cell and an Ia-positive non-T cell. J. Immunol., 124:437.

305 Swartz, A., S.L. Sutton, and R.K. Gershon 1982. Regulation of *in vitro* cytotoxic T lymphocyte generation. I. Evidence that killer cell precurcors differentiate to effector cells in two steps. J. Exp. Med., 155:783.

306 Symington, F.W. and H.-S. Teh 1980. A two-signal mechanism for the induction of cytotoxic T lymphocytes. Scand. J. Immunol. 12:1.

307 Symes, M., A. Riddell, E.J. Immelman, and J. Terblanche 1968. Immunologically competent cells in the treatment of malignant disease. Lancet 1:1054.

308 Takei, F., Levy, J.G., and Kilburn, D.G. 1976. In vitro induction of cytotoxicity

against syngeneic mastocytoma and its suppression by spleen and thymus cells from tumor-bearing mice. J. Immunol., 116:288.

309 Takei, F., J.G. Levy and D.G. Kilburn 1977. Characterization of suppressor cells in mice bearing syngeneic mastocytoma. J. Immunol. 118:412.

310 Takei, F., J.G. Levy and D.G. Kilburn 1978. Characterization of a soluble factor that specifically suppresses the in vitro generation of cells cytotoxic for syngeneic tumor cells in mice. J. Immunol., 120:1218.

311 Talmage, D.W., J.A. Woolnough, H. Hemmingsen, L. Lopez, and K.J. Lafferty 1977. Activation of cytotoxic T cells by nonstimulating tumor cells and spleen cell factor(s). Proc. Natl. Acad. Sci. USA, 74:4610.

312 Tanaka, Y., K. Sugamura, and Y. Hinuma 1982. Heterogeneity of allogeneic restriction of human cytotoxic T cell clones specific for Epstein Barr virus. J. Immunol., 128:1241.

313 Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto, and J. Hamuro 1983. Structure and expression of a cloned cDNA for human Interleukin-2. Nature 302:305.

314 Tees, R., and M.H. Schreier 1980. Selective reconstitution of nude mice with long-term cultured and cloned specific helper T cells. Nature 283:780.

315 Teh, H.-S. and S.-J. Teh 1980. Direct evidence for a two signal mechanism of cytotoxic T lymphocyte activation. Nature 285:163.

316 Tilden, A.B., and C.M. Balch 1982. A comparison of PGE2 effects on human suppressor cell function and on Interleukin 2 function. J. Immunol. 129:1269.

317 Ting, C.-C., D. Rodrigues and T. Igarashi 1979. Studies of the mechanisms for the induction of in vivo tumor immunity. III. Recruitment of host helper cells by donor T cells in adoptive transfer of cell-mediated immunity. J. Immunol., 122:1510.

318 Thoman, M.L., and W.O. Weigle 1982. Cell-mediated immunity in aged mice: An underlying lesion in IL2 synthesis. J. Immunol., 128:2358.

319 Thomas, W.R., P.L. Mottram and J.F.A.P. Miller 1982. Hapten specific T cell lines mediating delayed hyersensitivity to contact sensitizing agents. J. Exp. Med. 156:300.

320 Torres, B.A., W.L. Farrar and H.M. Johnson 1982. Interleukin 2 regulates Immune Interferon production by normal and suppressor cell cultures. J. Immunol. 128:2217.

321 Treves, A.J., and I.R. Cohen 1973. Recruitment of effector T lymphocytes against a tumor allograft by T lymphocytes sensitized *in vitro*. J. Nat. Cancer Inst., 51:1919.

322 Treves, A.J., I.R. Cohen and M. Feldman 1975. Immunotherapy of lethal metastases by lymphocytes sensitized against tumor cells in vitro. J. Nat. Can. Inst., 54:777.

323 Truitt, R.L., C. Shih, A.A. Rim, L.D. Tempelis and M.M. Bortin 1982. Alloimmunization of H-2-compatible donors for adoptive immunotherapy of leukemia: Role of H-2, Mls, and non-H-2 antigens. Prog. in Cancer Res. and Therapy, 22:21.

324 Uchiyama, T., D.L. Nelson, T.A. Fleisher, and T.A. Waldman 1981. A monoclonal

antibody (anti-Tac) reactive with activated and functionally mature human T cells. II. Expression of Tac antigen on activated cytotoxic killer cells, suppressor cells, and on one of the two types of helper T cells. J. Immuno. 126:1398.

325 Umetsu, D.T., S.P. Lerman, and G.J. Thorbecke 1979. Accessory cell requirements for lymphoma growth *in vitro* and in irradiated mice. Cell. Immunol., 42:139.

326 Umiel, T., and N. Trainin 1974. Immunological enhancement of tumor growth by syngeneic thymus-derived lymphocytes. Transplantation 18:244.

327 Unanue, E.R., 1980. Cooperation between mononuclear phagocytes and lymphocytes in immunity. N. Engl. J. Med., 303:977.

328 Urban, J.L., R.C. Burton, M. Holland, M.L. Kripke and H. Schreiber 1982. Mechanisms of syngeneic tumor rejection. Susceptibility of host-selected progressor variants to various immunologic effector cells. J. Exp. Med., 155:157.

329 Urban, J.L., and H. Schreiber 1983. Selection of macrophage-resistant progressor tumor variants by the normal host: Requirements for concomitant T cell-mediated immunity. J. Exp. Med. 157:642.

330 Vanky, F., T. Gorsky, Y. Gorsky, M.G. Masucci and E. Klein 1982. Lysis of tumor biopsy cells by autologous T lymphocytes activated in mixed cultures and propagated with T cell growth factor. J. Exp. Med., 155:83.

331 von Boehmer, H., and W. Hass 1981. H-2 restricted cytolytic and noncytolytic T cell clones: Isolation, specificity and functional analysis. Immunol. Rev. 54:25.

332 Vose, B.M., and G.D. Bonnard 1982. Human tumor antigens defined by cytotoxicity and proliferative responses of cultured lymphoid cells. Nature 296:359.

333 Vose, B.M. and G.D. Bonnard 1983. Limiting dilution analysis of the frequency of human T cells and large granular lymphocytes in response to interleukin 2. 1. The effect of lectin of the proliferative frequency and cytolytic activity of cultured lymphoid cells. J. Immunol. 130:687.

334 Wagner, H., 1973. Synergy during *in vitro* cytotoxic allograft responses. I. Evidence for cell interaction between thymocytes and peripheral T cells. J. Exp. Med., 138:1379.

335 Wagner, H., and M. Rollinghoff 1973. In vitro induction of tumor-specific immunity. I. Parameters of activation and cytotoxic reactivity of mouse lymphoid cells immunized in vitro against syngeneic and allogeneic plasma cell tumors. J. Exp. Med., 138:1.

336 Wagner, H., and M. Rollinghoff 1978. T-T-cell interactions during in vitro cytotoxic allograft responses. I. Soluble products from activated Ly1+ T cells trigger autonomously antigen-primed Ly23+ T cells to cell proliferation and cytolytic activity. J. Exp. Med., 148:1523.

337 Wagner, H., M. Rollinghoff, R. Schawaller, C. Hardt, and K. Pfizenmaier 1979. T cell-derived helper factor allows Lyt 123 thymocytes to differentiate into cytotoxic T lymphocytes. Nature, 280:405.

338 Wagner, H., C. Hardt, R. Bartlett, K. Pfizenmaier, M. Rollinghoff and K. Heeg 1980a. T-lymphocyte progenitors from thymus deficient (nu/nu) mice differentiate *in vitro* into H-2 restricted, hapten-specific cytotoxic effector

cells. Behring Inst. Mitt., 67:105.

339 Wagner, H., C. Hardt, K. Heeg, M. Rollinghoff and K. Pfizenmaier 1980b. T-cell-derived helper factor allows in vivo induction of cytotoxic T cells in nu/nu mice. Nature 284:278.

340 Wagner, H., C. Hardt, K. Heeg, K. Pfizenmaier, W. Solbach, R. Bartlett, H. Stockinger and M. Rollinghoff 1980c. T-T cell interactions during cytotoxic T Lymphocyte (CTL) responses: T cell derived helper factor (Interleukin 2) as a probe to analyze CTL responsiveness and thymic maturation of CTL progenitors. Immunol. Reviews, 51:215.

341 Wagner, H., M. Rollinghoff, K. Pfizenmaier, C. Hardt and G. Johnscher 1980d. T-T cell interactions during in vitro cytotoxic T lymphocyte (CTL) responses. II. Helper factor from activated Lyt 1+ T cells is rate limiting i) in T cell responses to nonimmunogenic alloantigen, ii) in thymocyte responses to allogeneic stimulator cells, and iii) recruits allo- or H-2 restricted CTL precursors from the Lyt 123+ subset. J. Immunol., 124:1058.

342 Wagner, H., C. Hardt, B.T. Rouse, M. Rollinghoff, P. Scheurich and K. Pfizenmaier 1982. Dissection of the proliferative and differentiative signals controlling murine cytotoxic T lymphocyte responses. J. Exp. Med., 155:1876.

343 Walker, C., F. Kristensen, F. Betteus, and A.L. Deweck 1983. Lymphokine regulation of activated G1 lymphocytes: 1. Prostaglandin E2 induced inhibition of Interleukin 2 production. J. Immunol. 130:1770.

344 Warren, H.S., J.A. Woolnough, C.P. Denaro and K.J. Lafferty 1979. Cytotoxic T cell responses to a syngeneic tumour: Conditions for primary activation in vitro and biological activity in vivo. Plenum Publishing Corporation, Function and Structure of the Immune System, Edited by W. Muller-Ruchholtz and H.K. Muller-Hermelink p.757.

345 Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. J. Exp. Med., 150:1510.

346 Watson, J., Aarden, L. A., Lefkovits, I. 1979. The purification and quantitation of helper T cell-replacing factors secreted by murine spleen cells activated by Concanavalin A. J. Immunol., 122:209.

347 Watson, J., L.A. Aarden, J. Shaw, and V. Paetkau 1979a. Molecular and quantitative analysis of helper T cell-replacing factors on the induction of antigen-sensitive B and T lymphocytes. J. Immunol., 122:1633.

348 Watson, J., S. Gillis, J. Marbrook, D. Mochizuki, and K.A. Smith 1979b. Biochemical and biological characterization of lymphocyte regulatory molecules. J. Exp. Med., 150:849.

349 Watson, J. and D. Mochizuki 1980. Purification and properties of T cell replacing factors. J. Supramolecular Structure, Suppl. 4:117.

350 Weiden. P.L., K.M. Sullivan, N. Flournoy, R. Storb, and D. Thomas 1981. Antileukemic effect of chronic graft-versus host disease: Contribution to improved survival after allogeneic marrow transplantation. New Eng. J. Med. 304:1529

351 Weiss, D. W. 1977. The questionable immunogenicity of certain neoplasms. Cancer Immunol. Immunother., 2:11.

352 Welsh, R.M. Jr. 1978. Mouse natural killer cells: Induction specificity, and function. J. Immunol., 121:1631.

353 Wepsic, H.T., B. Zbar, H.J. Rapp, and T. Borsos 1970a. Systemic transfer of tumor immunity: Delayed hypersensitivity and suppression of tumor growth. J. Nat. Cancer Inst., 44:955.

354 Wepsic, H.T., B.S. Kronman, B. Zbar, T. Borsos, and H.J. Rapp 1970b. Immunotherapy of an intramuscular tumor in strain-2 guinea pigs: Prevention of tumor growth by intradermal immunization and by systemic transfer of tumor immunity. J. Nat. Cancer Inst., 45:377.

355 Westcott, O., S. Dorsch, and B. Roser 1979. Adoptive immunotherapy of leukemia in the rat, without Graft-VS-Host complications. J. Immunol., 123:1478.

356 Wolos, J.A. and J.B. Smith 1982. Helper cells in the autologous mixed lymphocyte reaction. III. Production of helper factor(s) distinct from Interleukin 2. J. Exp. Med. 1807.

357 Woodruff, M., and B. Nolan 1963. Preliminary observations on treatment of advanced cancer by injection of allogeneic spleen cells. Lancet, 2:426.

358 Woodruff, M.F.A., M.O. Symes, and A.E. Stuart 1963. The effect of rat spleen cells on two transplanted mouse tumors. Br. J. Cancer, 17:320.

359 Woodruff, M.F.A., M.O. Symes and N.F. Anderson 1963b. The effect of intraperitoneal injection of thoracic duct lymphocytes from normal and immunized rats in mice innoculated with the Landschultz ascites tumor. Br. J. Cancer 17:482.

360 Yonemoto, R., and P. Terasaki 1972. Cancer immunotherapy with HLA compatible thoracic duct lymphocyte transplantation. Cancer 30:1438.

361 Yron, I., T.A. Wood Jr., P.J. Spiess and S.A. Rosenberg 1980. In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors. J. Immunol. 125:238.

362 Yu, A.M., H. Watts, N. Jaffe and R. Parkman 1977. Concomitant presence of tumor-specific cytotoxic and inhibitor lymphocytes in patients with osteogenic sarcoma. New Eng. J. Med. 297:121.

363 Zarling, J.M., H.E. Roberts, P.C. Raich, F.H. Bach and M.L. Bach 1978. Generation of cytotoxic T lymphocytes to autologous human leukemia cells by sensitization to pooled allogeneic normal cells. Nature 274:269.

364 Zarling, J.M., and F.H. Bach 1979. Continuous culture of T cells cytotoxic for autologous human leukaemia cells. Nature, 280:685.

365 Zinkernagel, R., and D.C. Doherty 1979. MHC-Restricted cytotoxic T cells: Studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity function and responsiveness. Adv. Immunol., 27:51.

PUBLICATIONS WHOLLY OR PARTLY BASED ON THIS THESIS

366 Gerhart, S., G. Mills, V. Monticone, and V. Paetkau 1976. Quantitative analysis of the proliferative activity induced in murine thymocytes by concanavalin A. J. Immunol., 117:1314.

367 Mills, G., V. Monticone, and V. Paetkau 1976. The role of macrophages in thymocyte mitogenesis. J. Immunol., 117:1325.

368 Mills, G.B. and V. Paetkau 1980. Generation of cytotoxic lymphocytes to syngeneic tumor by using costimulator (Interleukin 2). J. Immunol. 125:1897.

369 Mills, G.B., G. Carlson and V. Paetkau 1980. Generation of cytotoxic lymphocytes to syngeneic tumors using costimulator (IL2): In vivo activity. J. Immunol. 125:1904.

370 Paetkau, V., G. Mills, S. Gerhart, and V. Monticone 1976. Proliferation of murine thymic lymphocytes in vitro is mediated by the concanavalin A-induced release of a lymphokine (costimulator). J. Immunol. 117:1320.

371 Paetkau, V., J. Shaw, B. Caplan, G.B. Mills and K.-C. Lee 1980a. Interleukin 2 in cell-mediated immune responses. J. Supramol. Structure 13:271.

372 Paetkau, V., J. Shaw, G. Mills and B. Caplan 1980b. Cellular origins and targets of costimulator (IL2). Immunol. Revs. 51:157.

373 Paetkau, V., G.B. Mills, and R.C. Bleackley 1982. Enhancement of anti-tumor immune responses with Interleukin 2. Prog. in Cancer Res. and Therapy, 22:147.

374 Shaw, J., V. Monticone, G. Mills, and V. Paetkau 1978a. Effects of costimulator on immune responses in vitro. J. Immunol., 120:1974.

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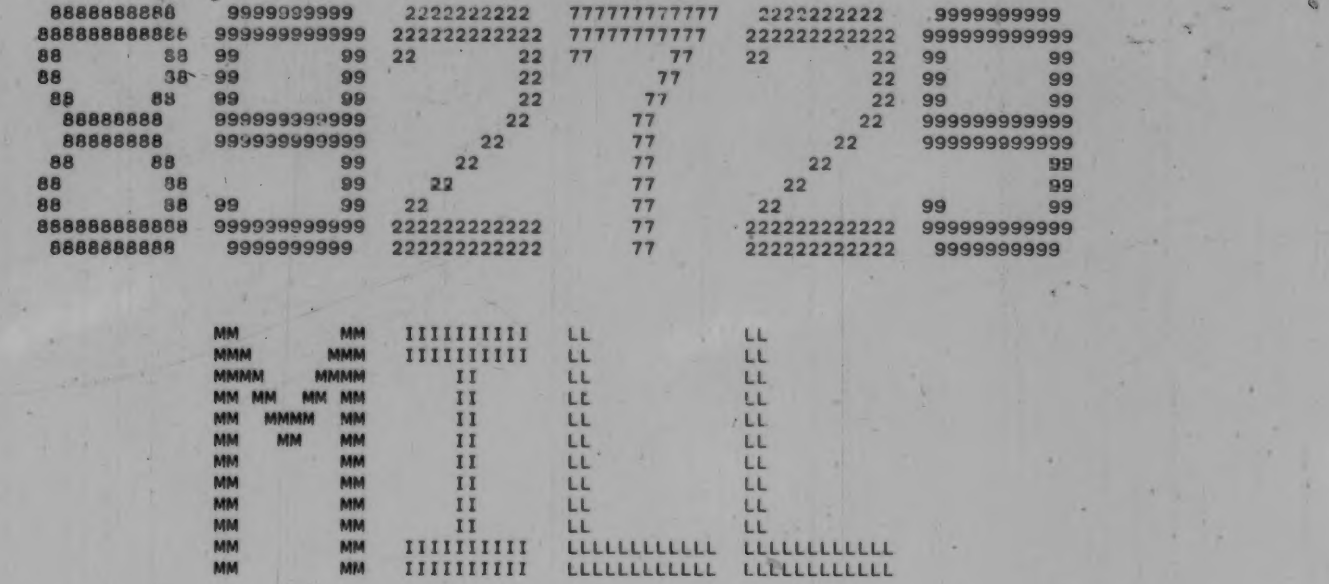
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